PRIORITY DOCUMENT

SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

RECEIVED
16 MAR 2004
WIPO PCT

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

IB/04/575

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

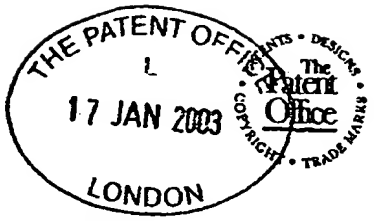
-registration under the Companies Act does not constitute a new legal entity but merely jects the company to certain additional company law rules.

Signed

Dated 13 January 2004

Patents Form 1/77

Patents Act 1977
(Rule 16)



203003 E778046-5 002246
P01/7700 0.00-0301121.0

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP10 8QQ

1. Your reference — P013245GB AAW

2. Patent application number *(The Patent Office will fill in this part)* — 0301121.0 ✓ — 17 JAN 2003 ✓

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Danisco A/S
Langebrogade 1
PO Box 17
DK-1001 Copenhagen K
Denmark

Patents ADP number *(if you know it)* 6608730002

If the applicant is a corporate body, give the country/state of its incorporation — Denmark

4. Title of the invention — METHOD

5. Name of your agent *(if you have one)* — D Young & Co

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

21 New Fetter Lane
London
EC4A 1DA

Patents ADP number *(if you know it)* — 59006 ✓

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

Yes

Patents Form 1/77

Patents Form 1/77

9. Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	0
Description	60
Claim(s)	3
Abstract	1 DMC
Drawing(s)	14 + 2

10. If you are also filing any of the following, state how many against each item.

Priority documents	0
Translations of priority documents	0
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	0
Request for preliminary examination and search *(Patents Form 9/77)*	0
Request for substantive examination *(Patents Form 10/77)*	0
Any other documents *(please specify)*	None

11. I/We request the grant of a patent on the basis of this application.

Signature D Young & Co Date 17 January 2003

D Young & Co (Agents for the Applicants)

12. Name and daytime telephone number of person to contact in the United Kingdom — David Alcock — 023 8071 9500

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 08459 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

METHOD

FIELD OF INVENTION

The present invention relates to a method for the *in situ* production of two emulsifiers within a foodstuff by use of a lipid:carbohydrate acyltransferase.

The present invention further relates to a method for the *in situ* production of a carbohydrate ester within a foodstuff by use of a lipid:carbohydrate acyltransferase.

TECHNICAL BACKGROUND

WO00/05396 teaches a process for preparing a foodstuff comprising an emulsifier, wherein food material is contacted with an enzyme such that an emulsifier is generated by the enzyme from a fatty acid ester and a second functional ingredient is generated from a second constituent. WO00/05396 teaches the use of a lipase or esterase enzyme. Nowhere in WO00/05396 is the specific use of a lipid:carbohydrate acyltransferase taught.

The use of lipases (EC. 3.1.1.x) in the food and/or feed industries, for example in foods and/or feeds comprising cereals and, in particular in bread production, has been considered. For instance, in EP 0 585 988 it is claimed that lipase addition to dough resulted in an improvement in the antistaling effect. It is suggested that a lipase obtained from *Rhizopus arrhizus* when added to dough can improve the quality of the resultant bread when used in combination with shortening/fat. WO94/04035 teaches that an improved softness can be obtained by adding a lipase to dough without the addition of any additional fat/oil to the dough. Castello, P. ESEGP 89-10 Dec. 1999 Helsinki, shows that exogenous lipases can modify bread volume.

The substrate for lipases in wheat flour is 1.5-3% endogenous wheat lipids, which are a complex mixture of polar and non-polar lipids. The polar lipids can be divided into glycolipids and phospholipids. These lipids are built up of glycerol esterified with two

fatty acids and a polar group. The polar group contributes to surface activity of these lipids. Enzymatic cleavage of one of the fatty acids in these lipids leads to lipids with a much higher surface activity. It is well known that emulsifiers, such as DATEM, with high surface activity are very functional when added to dough.

However, it has also been found that under certain conditions the use of lipases (E.C. 3.1.1.X) in foodstuffs, particularly dough, may have detrimental consequences, such as the production of off-flavours, a detrimental impact on yeast activity, and/or a negative effect on bread volume. The negative effect on bread volume is often called overdosing. Overdosing can lead to a decrease in gluten elasticity which results in a dough which is too stiff and thus results in reduced volumes. In addition, or alternatively, such lipases can degrade shortening, oil or milk fat added to the dough. The disadvantages associated with the use of lipases may be caused by the build-up of free fatty acids released from the lipids.

Lipid:cholesterol acyltransferases have been known for some time (see for example Buckley – Biochemistry 1983, 22, 5490-5493). In particular, glycerophospholipid:cholesterol acyl transferases (GCATs) have been found, which like the plant and/or mammalian lecithin:cholesterol acyltransferases (LCATs), will catalyse fatty acid transfer between phosphatidylcholine and cholesterol. However, these enzymes have been found to be highly selective for specific acyl acceptors, namely cholesterol (see Buckley 1983 supra).

Upton and Buckley (TIBS 20, May 1995 p 178-179) and Brumlik and Buckley (J. of Bacteriology Apr. 1996 p 2060-2064) teach a lipase/acyltransferase from *Aeromonas hydrophila* which has the ability to carry out acyl transfer to alcohol acceptors in aqueous media. Nowhere in this document it is taught or suggested that the enzyme may be capable of transferring the acyl group to a carbohydrate acceptor.

SUMMARY ASPECTS OF THE PRESENT INVENTION

According to a first aspect of the present invention there is provided a method for the *in situ* production of two emulsifiers in a foodstuff, wherein the method comprises the step of adding to the foodstuff a lipid:carbohydrate acyltransferase.

In a further aspect, the present invention provides a method for the *in situ* production of a carbohydrate ester in a foodstuff, wherein the method comprises the step of adding a lipid:carbohydrate acyltransferase to the foodstuff.

In another aspect, the present invention provides a method for the *in situ* production of a carbohydrate ester together with an emulsifier in a foodstuff, wherein the method comprises the step of adding a lipid:carbohydrate acyltransferase to the foodstuff.

According to a further aspect of the present invention there is provided a method for the production of a foodstuff comprising two emulsifiers, wherein the method comprises the step of adding to the foodstuff a lipid:carbohydrate acyltransferase.

In a further aspect, the present invention provides a method for the production of a foodstuff comprising a carbohydrate ester, wherein the method comprises the step of adding a lipid:carbohydrate acyltransferase to the foodstuff.

In another aspect, the present invention provides a method for the production of a foodstuff comprising a carbohydrate ester and an emulsifier, wherein the method comprises the step of adding a lipid:carbohydrate acyltransferase to the foodstuff.

In another aspect, the present invention provides use of a lipid:carbohydrate acyltransferase to prepare from a food material a foodstuff comprising at least two emulsifiers, wherein the two emulsifiers are generated from constituents of the food material by the lipid:carbohydrate acyltransferase.

In a further aspect, the present invention provides use of a lipid:carbohydrate acyltransferase to prepare from a food material a foodstuff comprising a carbohydrate

ester, wherein the carbohydrate ester is generated from constituents of the food material by the lipid:carbohydrate acyltransferase.

In another aspect, the present invention provides use of a lipid:carbohydrate acyltransferase to prepare from a food material a foodstuff comprising at least a carbohydrate ester and a further emulsifier, wherein the carbohydrate ester and the emulsifier are generated from constituents of the food material by the lipid:carbohydrate acyltransferase.

In a further aspect, the present invention further provides a foodstuff obtainable by, preferably obtained by, the method according to the present invention.

DETAILED ASPECTS OF THE PRESENT INVENTION

The term "lipid:carbohydrate acyltransferase" as used herein means an enzyme which as well as having lipase activity (generally classified as E.C. 3.1.1.x in accordance with the Enzyme Nomenclature Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology) also has acyltransferase activity (generally classified as E.C. 2.3.1.x), whereby the enzyme is capable of transferring an acyl group from a lipid to a carbohydrate.

Preferably, the lipid substrate upon which the lipid:carbohydrate acyltransferase according to the present invention acts is one or more of the following lipids: a phospholipid, such as phosphatidylcholine for example, a triacylglyceride or a diglyceride. This lipid substrate may be referred to herein as the "lipid acyl donor". The term phosphatidylcholine as used herein is synonymous with the term lecithin and these terms may be used herein interchangeably.

For some aspects, preferably the lipid:carbohydrate acyltransferase according to the present invention is incapable or substantially incapable of acting on a triglyceride and/or on a 1-monoglyceride.

Preferably the lipid substrate is a food lipid, that is to say a lipid component of a foodstuff.

Suitably, the lipid substrate or lipid acyl donor may be one or more lipids present in one or more of the following substrates: fats, including lard, tallow and butter fat; oils including oils extracted from or derived from palm oil, sunflower oil, soya bean oil, safflower oil, cotton seed oil, ground nut oil, corn oil, olive oil, peanut oil, coconut oil, and rape seed oil. Suitably, the lipid substrate or lipid acyl donor may be lecithin from soya, rape seed or egg yolk.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of from 8 to 22 carbons.

For some aspects of the present invention, the lipid may be selected from lipids having a fatty acid chain length of from 16 to 22 carbons, more preferably of from 16 to 20 carbons.

For some aspect of the present invention, the lipid may be selected from lipids having a fatty acid chain length of no greater than 14 carbons, suitably from lipids having a fatty acid chain length of from 4 to 14 carbons, suitably 4 to 10 carbons, suitably 4 to 8 carbons.

Suitably, the lipid:carbohydrate acyltransferase according to the present invention may exhibit one or more of the following lipase activities: triacylglycerol lipase activity (E.C. 3.1.1.3), phospholipase A2 activity (E.C. 3.1.1.4) or phospholipase A1 activity (E.C. 3.1.1.32).

For some aspects, the lipid:carbohydrate acyltransferase according to the present invention may suitably not exhibit triacylglycerol lipase activity (E.C. 3.1.1.3).

Suitably, the carbohydrate utilised by the lipid:carbohydrate acyltransferase according to the present invention as a "acyl acceptor" may be one or more of the following: a monosaccharide, a disaccharide, an oligosaccharide, or a polysaccharide. Preferably, the carbohydrate is one or more of the following: glucose, fructose, anhydrofructose, maltose, lactose, galactose, xylose, xylooligosaccharides, arabinose, maltooligosaccharides, tagatose or sucrose.

Suitably, the carbohydrate "acyl acceptor" may be found naturally within the foodstuff. Alternatively, the carbohydrate may be added to the foodstuff. When it is the case that the carbohydrate is added to the foodstuff, the carbohydrate may be added before, simultaneously with, and/or after the addition of the lipid:carbohydrate acyltransferase according to the present invention.

Preferably, the lipid:carbohydrate acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to a carbohydrate acyl acceptor to form a new ester, i.e. a carbohydrate ester; and

(ii) the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

Preferably, X of the GDSX motif is L. Thus, preferably the enzyme according to the present invention comprises the amino acid sequence motif GSDL.

The GDSX motif is comprised of four conserved amino acids. Preferably, the serine within the motif is a catalytic serine of the lipid:carbohydrate acyltransferase enzyme. Suitably, the serine of the GDSX motif may be in a position corresponding to Ser-16 in *Aeromonas hydrophila* lipolytic enzyme taught in Brumlik & Buckley (Journal of Bacteriology Apr. 1996, Vol. 178, No. 7, p 2060-2064).

To determine if a protein has the GDSX motif according to the present invention, the sequence is preferably compared with the hidden markov model profiles (HMM profiles) of the pfam database. A hidden markov model profile is based on a manually verified multiple sequence alignment of a representative set of sequences comprising a protein domain family, and is used for alignment purposes. A positive match with the hidden markov model profile (HMM profile) of the pfam00657.6 domain family indicates the presence of the GDSL or GDSX domain according to the present invention. For a detailed explanation of the theory and implementation of hidden markov models see Durbin *et al* 1998 Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids, Cambridge Uni. Press, ISBN: 0-521-62051-4. For pfam alignment and scoring procedures see [A. Bateman *et al*: Nucleic Acids Research, 30(1):276-280, 2002]. The pfam database can be accessed through the internet and is currently available at one of the following web pages:
http://www.sanger.ac.uk/Software/Pfam/index.shtml
http://pfam.wustl.edu/
http://www.cgr.ki.se/Pfam/
http://pfam.jouy.inra.fr/

The pfam00657.6 GDSX domain is a unique identifier which distinguishes proteins possessing this domain from other enzymes.

The pfam00657.6 consensus sequence is presented in Figure 1 as SEQ ID No. 1.

Preferably, the lipid:carbohydrate acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to a carbohydrate acyl acceptor to form a new ester, i.e. a carbohydrate ester;

(ii) the enzyme comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S;

(iii) the enzyme comprises His-309 or comprises a histidine residue at a position corresponding to His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2).

Preferably, the X of the GDSX motif is L.

In SEQ ID No. 2 the first 18 amino acid residues form a signal sequence. His-309 of the full length sequence, that is the protein including the signal sequence, equates to His-291 of the mature part of the protein, i.e. the sequence without the signal sequence.

Preferably, the lipid:carbohydrate acyltransferase enzyme according to the present invention comprises the following catalytic triad: Ser-34, Asp-134 and His-309 or comprises a serine residue, an aspartic acid residue and a histidine residue, respectively, at positions corresponding to Ser-34, Asp-134 and His-309 in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2). As stated above, in the sequence shown in SEQ ID No. 2 the first 18 amino acid residues form a signal sequence. Ser-34, Asp-134 and His-309 of the full length sequence, that is the protein including the signal sequence, equate to Ser-16, Asp-116 and His-291 of the mature part of the protein, i.e. the sequence without the signal sequence. In the pfam00657.6 consensus sequence, as given in Figure 1 (SEQ ID No. 1) the active site residues correspond to Ser-7, Asp-157 and His-348.

Preferably, the lipid:carbohydrate acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to a carbohydrate acyl acceptor to form a new ester, i.e. a carbohydrate ester; and

(ii) the enzyme comprises at least Gly-32, Asp-33, Ser-34, Asp-134 and His-309 or comprises glycine, aspartic acid, serine, aspartic acid and histidine residues at positions corresponding to Gly-32, Asp-33, Ser-34,

Asp-134 and His-309, respectively, in the *Aeromonas hydrophila* lipolytic enzyme shown in Figure 2 (SEQ ID No. 2).

Suitably, the lipid:carbohydrate acyltransferase enzyme according to the present invention may be characterised using the following criteria:

(i) the enzyme possesses acyl transferase activity which may be defined as ester transfer activity whereby the acyl part of an original ester bond of a lipid acyl donor is transferred to a carbohydrate acyl acceptor to form a new ester, i.e. a carbohydrate ester; and

(ii) the enzyme comprises Gly-5, Asp-6, Ser-7, Asp-157 and His-348 or comprises glycine, aspartic acid, serine, aspartic acid and histidine residues at positions corresponding to Gly-5, Asp-6, Ser-7, Asp-156 and His-348, respectively, in the pfam00657.6 consensus sequence shown in Figure 1 (SEQ ID No. 1).

Suitably, the lipid:carbohydrate acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from organisms from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces, Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium, Ralstonia, Xanthomonas* and *Candida.*

Suitably, the lipid:carbohydrate acyltransferase enzyme according to the present invention may be obtainable, preferably obtained, from one or more of the following organisms: *Aeromonas hydrophila, Aeromonas salmonicida, Streptomyces coelicolor, Mycobacterium, Streptococcus pyogenes, Lactococcus lactis, Streptococcus pyogenes, Streptococcus thermophilus, Lactobacillus helveticus, Desulfitobacterium dehalogenans, Bacillus sp, Campylobacter jejuni, Vibrionaceae, Xylella fastidiosa, Sulfolobus solfataricus, Saccharomyces cerevisiae, Aspergillus terreus, Schizosaccharomyces pombe, Listeria innocua, Listeria monocytogenes, Neisseria meningitidis, Mesorhizobium loti, Ralstonia solanacearum, Xanthomonas campestris, Xanthomonas axonopodis* and *Candida parapsilosis.*

Suitably, the lipid:carbohydrate acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(i) the amino acid sequence shown as SEQ ID No. 2 (see Figure 2)

(ii) the amino acid sequence shown as SEQ ID No. 3 (see Figure 3)

(iii) the amino acid sequence shown as SEQ ID No. 4 (see Figure 4)

(iv) the amino acid sequence shown as SEQ ID No. 5 (see Figure 5)

(v) the amino acid sequence shown as SEQ ID No. 6 (see Figure 6)

(vi) the amino acid sequence shown as SEQ ID No. 12 (see Figure 14)

(vii) an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6 or SEQ ID No. 12.

Suitably the nucleotide sequence may have 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11 or SEQ ID No. 13.

For the purposes of the present invention, the degree of identity is based on the number of sequence elements which are the same. The degree of identity in accordance with the present invention may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, US53711) (Needleman & Wunsch (1970), J. of Molecular Biology 48, 443-45) using the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Suitably the lipid:carbohydrate acyltransferase enzyme according to the present invention comprises an amino acid sequence which has 80% or more, preferably 85% or more, more preferably 90% or more and even more preferably 95% or more identity

with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6 or SEQ ID No. 12.

Suitably, the lipid:carbohydrate acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(a) an amino acid sequence shown as amino acid residues 1-100 of SEQ ID No. 2;

(b) an amino acid sequence shown as amino acids residues 101-200 of SEQ ID No. 2;

(c) an amino acid sequence shown as amino acid residues 201-300 of SEQ ID No. 2; or

(d) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(c) above.

Suitably, the lipid:carbohydrate acyltransferase enzyme according to the present invention comprises one or more of the following amino acid sequences:

(a) an amino acid sequence shown as amino acid residues 28-39 of SEQ ID No. 2;

(b) an amino acid sequence shown as amino acids residues 77-88 of SEQ ID No. 2;

(c) an amino acid sequence shown as amino acid residues 126-136 of SEQ ID No. 2;

(d) an amino acid sequence shown as amino acid residues 163-175 of SEQ ID No. 2;

(e) an amino acid sequence shown as amino acid residues 304-311 of SEQ ID No. 2; or

(f) an amino acid sequence which has 75% or more, preferably 85% or more, more preferably 90% or more, even more preferably 95% or more identity to any one of the amino acid sequences defined in (a)-(e) above.

Suitably, the lipid:carbohydrate acyltransferase enzyme according to the present invention may comprise an amino acid sequence produced by the expression or one or more of the following nucleotide sequences:

(a) the nucleotide sequence shown as SEQ ID No. 7 (see Figure 9);

(b) the nucleotide sequence shown as SEQ ID No. 8 (see Figure 10);

(c) the nucleotide sequence shown as SEQ ID No. 9 (see Figure 11);

(d) the nucleotide sequence shown as SEQ ID No. 10 (see Figure 12);

(e) the nucleotide sequence shown as SEQ ID No. 11 (see Figure 13);

(f) the nucleotide sequence shown as SEQ ID No. 13 (see Figure 15);or

(g) a nucleotide sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 7, SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11 or SEQ ID No. 13.

Preferably, the emulsifier and/or carbohydrate ester produced in accordance with the present invention is produced without increasing or substantially without increasing the free fatty acids in the foodstuff. The term "without increasing or without substantially increasing the free fatty acids" as used herein means that preferably the lipid:carbohydrate acyltransferase according to the present invention has 100% transferase activity (i.e. transfers 100% of the acyl groups from an acyl donor onto the carbohydrate acyl acceptor, with no hydrolytic activity); however, the enzyme may transfer less than 100% of the acyl groups present in the lipid acyl donor to the carbohydrate. In which case, preferably the acytransferase activity accounts for at least 5%, more preferably at least 10%, more preferably at least 20%, more preferably at least 30%, more preferably at least 40%, more preferably 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90% and more preferably at least 98% of the total enzyme activity. The % transferase activity (i.e. the transferase activity as a percentage of the total enzymatic activity) may be determined by the following protocol:

Protocol for the determination of % acyltransferase activity:

A foodstuff to which a lipid:carbohydrate acyltransferase according to the present invention has been added may be extracted following the enzymatic reaction with $CHCl_3$:CH_3OH 2:1 and the organic phase containing the lipid material is isolated and analysed by GLC and HPLC according to the procedure detailed hereinbelow. From the GLC and HPLC analyses the amount of free fatty acids and sugar esters are determined. A control foodstuff to which no enzyme according to the present invention has been added, is analysed in the same way.

Calculation:

From the results of the GLC and HPLC analyses the increase in free fatty acids and sugar esters can be calculated:

Δ % fatty acid = % Fatty acid(enzyme) - % fatty acid(control)

Δ % sugar ester = % sugar ester(enzyme) - % sugar ester (control)

The transferase activity is calculated as a percentage of the total enzymatic activity:

$$\% \text{ transferase activity} = \frac{\Delta \% \text{ sugar ester}/(\text{Mv sugar ester}) \times 100}{\Delta \% \text{ sugar ester}/(\text{Mv sugar ester}) + \Delta \% \text{ fatty acid}/(\text{Mv fatty acid})}$$

where:

Mv sugar ester = average molecular weight of the sugar esters

Mv fatty acid = average molecular weight of the fatty acids

If the free fatty acids are increased in the foodstuff they are preferably not increased to a significant degree. By this we mean, that the increase in free fatty acid does not adversely affect the quality of the foodstuff.

Thus, for some aspects of the present invention the overall level of free fatty acids in the foodstuff does not increase or increases only to an insignificant degree. This is in sharp contradistinction to the situation when lipase (E.C. 3.1.1.x) are used to produce emulsifiers *in situ*. In particular, the use of lipases can result in an increased amount of free fatty acid in the foodstuff, which can be detrimental. In accordance with the present invention, the accumulation of free fatty acids is reduced and/or eliminated when compared with the amount of free fatty acids which would have been accumulated had a lipase enzyme, in particular a lipolytic enzyme such as Grindamyl Exel 16 (Danisco A/S, Denmark) or Lipopan F (Novozymes A/S, Denmark), been used in place of the lipid:carbohydrate acyltransferase in accordance with the present invention.

The term "*in situ*" as used herein means that the carbohydrate ester and/or the emulsifier(s) are produced within the foodstuff or fraction of the foodstuff. This contrasts the situation where the carbohydrate ester and/or the emulsifier(s) are

produced separately of the foodstuff and are added as formed products to the foodstuff during preparation of the same. In other words, the term "*in situ*" as used herein means that by the addition of the lipid:carbohydrate acyltransferase enzyme according to the present invention to a foodstuff, or to the food ingredients/materials constituting the foodstuff, a carbohydrate ester and/or an emulsifier may be produced from components of the foodstuff. Suitably, the components of the foodstuff may be the substrate(s) for the enzyme. If necessary, the components of the foodstuff may be supplemented by addition of one or more further components which further components may be the same as those present in the foodstuff or may be additional to those components already present in the foodstuff.

Preferably, the lipid:carbohydrate acyltransferase according to the present invention is capable of transferring an acyl group from a lipid to a carbohydrate when present in a polar environment, preferably in an aqueous environment, preferably a water containing foodstuff. Suitably, however the aqueous environment may be an aqueous buffer. The term "aqueous environment" as used herein means preferably an environment which is absent an organic solvent, preferably absent a polar organic solvent. In particular, the term "aqueous environment" may refer to an environment to which no exogenous organic solvents, preferably no polar organic solvents, have been added. The term organic solvent as used herein does not encompass food oils that are high in non-polar lipids. Suitably, the aqueous environment according to the present invention may comprise less than 30% by volume organic solvents, more preferably less than 15% by volume organic solvents, more preferably less than 5%. Suitably the foodstuff may comprise between 1 and 5% organic solvent, for example ethanol. However, when the foodstuff comprises such an organic solvent, preferably it is produced endogenously within the foodstuff. That is to say, when the foodstuff comprises such an organic solvent, preferably the organic solvent is not an exogenous organic solvent.

The term "foodstuff" as used herein means a substance which is suitable for human and/or animal consumption.

Suitably, the term "foodstuff" as used herein may mean a foodstuff in a form which is ready for consumption. Alternatively or in addition, however, the term foodstuff as used herein may mean one or more food materials which are used in the preparation of a foodstuff. By way of example only, the term foodstuff encompasses both baked goods produced from dough as well as the dough used in the preparation of said baked goods.

In a preferred aspect the present invention provides a foodstuff as defined above wherein the foodstuff is selected from one or more of the following: baked goods, including breads, cakes, sweet dough products, laminated doughs, liquid batters, muffins, doughnuts, biscuits, crackers and cookies; confectionery, including chocolate, candies, caramels, halawa, gums, including sugar free and sugar sweetened gums, bubble gum, soft bubble gum, chewing gum and puddings; frozen products including sorbets, preferably frozen dairy products, including ice cream and ice milk; dairy products, including cheese, butter, milk, coffee cream, whipped cream, custard cream, milk drinks and yoghurts; mousses, whipped vegetable creams, meat products, including processed meat products; edible oils and fats, aerated and non-aerated whipped products, oil-in-water emulsions, water-in-oil emulsions, margarine, shortening and spreads including low fat and very low fat spreads; dressings, mayonnaise, dips, cream based sauces, cream based soups, beverages, spice emulsions, sauces and mayonnaise; eggs, egg-based products, including but not limited to mayonnaise, salad dressings, sauces, ice creams, egg powder, modified egg yolk and products made therefrom.

Suitably the foodstuff in accordance with the present invention may be a "fine foods", including cakes, pastry, confectionery, chocolates, fudge and the like.

In one aspect the foodstuff in accordance with the present invention may be a dough product or a baked product, such as a bread, a fried product, a snack, cakes, pies, brownies, cookies, noodles, snack items such as crackers, graham crackers, pretzels, and potato chips, and pasta.

In a further aspect, the foodstuff in accordance with the present invention may be a plant derived food product such as flours, pre-mixes, oils, fats, cocoa butter, coffee whitener, salad dressings, margarine, spreads, peanut butter, shortenings, ice cream, cooking oils.

In another aspect, the foodstuff in accordance with the present invention may be a dairy product, including butter, milk, cream, cheese such as natural, processed, and imitation cheeses in a variety of forms (including shredded, block, slices or grated), cream cheese, ice cream, frozen desserts, yoghurt, yoghurt drinks, butter fat, anhydrous milk fat, other dairy products. The enzyme according to the present invention may improve fat stability in dairy products. In addition, the enzyme according to the present invention may be used to prevent and/or reduce the production of a soapy taste in cheese, which soapy taste is typically associated with the production of long chain fatty acids.

In another aspect, the foodstuff in accordance with the present invention may be a food product containing animal derived ingredients, such as processed meat products, cooking oils, shortenings.

In one aspect, the foodstuff may be selected from one or more of the following: eggs, egg-based products, including mayonnaise, salad dressings, sauces, ice cream, egg powder, modified egg yolk and products made therefrom.

Preferably the foodstuff according to the present invention is a water containing foodstuff. Suitably the foodstuff may be comprised of 10-98% water, suitably 14–98%, suitably of 18-98% water, suitably of 20-98%, suitably of 40-98%, suitably of 50-98%, suitably of 70-98%, suitably of 75-98%.

For some aspects, preferably the foodstuff in accordance with the present invention is not a pure plant derived oil, such as olive oil, sunflower oil, peanut oil, rapeseed oil for instance. For the avoidance of doubt, in some aspects of the present invention the foodstuff according to the present invention may comprise an oil, but preferably the

foodstuff is not primarily composed of oil or mixtures of oil. For some aspects, preferably the foodstuff comprises less than 95% lipids, preferably less than 90% lipids, preferably less than 85%, preferably less than 80% lipids. Thus, for some aspects of the present invention oil may be a component of the foodstuff, but preferably the foodstuff is not a oil *per se*.

The claims of the present invention are to be construed to include each of the foodstuffs listed above.

Preferably, when it is the case that two emulsifiers are produced, one of the emulsifiers is a carbohydrate ester.

Suitably, the carbohydrate ester produced in accordance with the present invention may be an oligosaccharide ester.

Suitably, the carbohydrate ester produced in accordance with the present invention may be a monosaccharide ester or a disaccharide ester.

Preferably, the carbohydrate ester produced in accordance with the present invention is one or more of the following: glucose ester, fructose ester, anhydrofructose ester, maltose ester, lactose ester, galactose ester, xylose ester, xylooligosacchride ester, arabinose ester, maltooligosaccharide ester, tagatose ester or sucrose ester.

Preferably, the carbohydrate ester produced in accordance with the present invention is one or more of the following: a carbohydrate mono-ester, a sugar mono-ester, an oligosaccharide mono-ester, a trisaccharide mono-ester, a disaccharide mono-ester, a monosaccharide mono-ester, a glucose mono-ester, a fructose mono-ester, anhydrofructose mono-ester, maltose mono-ester, lactose mono-ester, galactose mono--ester, xylose mono-ester, xylooligosacchride mono-ester, arabinose mono-ester, maltooligosaccharide mono-ester, tagatose mono-ester or sucrose mono-ester.

Preferably, the formation of the carbohydrate ester in accordance with the present invention is independent of UDP-glucose.

Preferably, the foodstuff according to the present invention does not comprise UDP-glucose, or only comprises UDP-glucose in insignificant amounts.

Where the first emulsifier is a carbohydrate ester, the second emulsifier may be for example one or more of the following: a diglyceride, a monoglyceride, such as 1-monoglyceride or a lysophosphatidylcholine. The second emulsifier is preferably produced from the lipid acyl donor following removal of one or more acyl groups from said lipid acyl donor. The term lysophosphatidylcholine as used herein is synonymous with the term lysolecithin and these terms may be used herein interchangeably.

The lipase and acyltransferase activity of an enzyme may be evaluated using the following assays. In this way, a lipid:carbohydrate acyltransferase having the enzyme characteristics defined herein may be obtained.

Lipase assay based on tributyrin as substrate (LIPU): Lipase activity based on tributyrin may be measured according to Food Chemical Codex, Forth Edition, National Academy Press, 1996, p 803, with the modification that the sample is dissolved in deionized water instead of glycine buffer, and the pH stat set point is 5.5 instead of 7. 1 LIPU is defined as the quantity of enzyme which can liberate 1 µmol butyric acid per minute under assay conditions.

Lipid:carbohydrate acyltransferase assay for the determination of a lipid:carbohydrate acyltransferase unit, LATU(Gl): Lipid acyl transferase activity is defined as the amount of µmol glucose ester formed per minute from lecithin as donor and glucose as acceptor molecule under assay conditions using the following procedure: Substrate :0,6% Avanti phospholipid(95% PC), 0.4 % glucose, 0.05M HEPES buffer pH 7 and 5 mM $CaCl_2$. Glucose is dissolved in buffer and Avanti phospholipid is dispersed by heating to 40°C and homogenized on a Turrax mixer for 10 seconds. 1 ml substrate is thermostated to 30 °C for 5 minutes. 100 µl of enzyme solution is added. After 10

minutes the reaction is stopped by adding 0.1 ml 1M HCl. The lipid components are extracted into an organic phase by adding 1 ml $CHCl_3$:CH_3OH 2:1. 500 µl of the organic phase is transferred to a 10 ml flask and evaporated at 60°C in a stream of nitrogen. The amount of carbohydrate ester formed is determined by GLC analyses, using glucose monooleate (>95%) as standard.

Preferably the method and/or use according to the present invention may be carried out in foodstuff at a temperature of 15-60°C, preferably at a temperature of 20-60°C. For some aspects, for example in dough, preferably the temperature of the food during which the acyltransferase reaction takes place is between 20 and 40°C. For other aspects, for example with regard to dairy products, such as cheese, the temperature of the food may suitably be between 30°C and 60°C. In yet other aspects, for example with regard to mayonnaise, the temperature of the food may suitably be between 20 and 40°C, more preferably between 25 and 30°C.

Preferably, the carbohydrate ester produced according to the present invention comprises less than 5 wt % of the foodstuff.

Preferably, the carbohydrate ester produced according to the present invention comprises from 0.01 to 4 wt % of the foodstuff.

Preferably, the carbohydrate ester produced according to the present invention comprises from 0.01 to 2 wt % of the foodstuff.

Preferably, the carbohydrate ester produced according to the present invention comprises from 0.01 to 1 wt % of the foodstuff.

Preferably, the carbohydrate ester produced according to the present invention comprises from 0.01 to 0.5 wt % of the foodstuff.

Preferably, the carbohydrate ester produced according to the present invention comprises from 0.01 to 0.3 wt % of the foodstuff.

Preferably, the total amount of emulsifier produced *in situ* according to the present invention comprises less than 5 wt % of the foodstuff.

Preferably, the total amount of emulsifier produced *in situ* according to the present invention comprises from 0.01 to 4 wt % of the foodstuff.

Preferably, the total amount of emulsifier produced *in situ* according to the present invention comprises from 0.01 to 2 wt % of the foodstuff.

Preferably, the total amount of emulsifier produced *in situ* according to the present invention comprises from 0.01 to 1 wt % of the foodstuff.

Preferably, the total amount of emulsifier produced *in situ* according to the present invention comprises from 0.01 to 0.5 wt % of the foodstuff.

Preferably, the total amount of emulsifier produced *in situ* according to the present invention comprises from 0.01 to 0.3 wt % of the foodstuff.

The enzyme according to the present invention is preferably not immobilised, and preferably not immobilised on a solid support.

Suitably, the method according to the present invention includes inactivating or denaturing the enzyme to provide a foodstuff comprising the enzyme in an inactive or denatured form. Suitably the enzyme may be denatured by either baking or by pasteurisation.

Preferably the enzyme according to the present invention is present in an inactive form or in a denatured form in the foodstuff.

The enzyme according to the present invention may be used with one or more other suitable food grade enzymes. Thus, it is within the scope of the present invention that,

in addition to the enzyme of the invention, at least one further enzyme is added to the foodstuff. Such further enzymes include starch degrading enzymes such as endo- or exoamylases, pullulanases, debranching enzymes, hemicellulases including xylanases, cellulases, oxidoreductases, e.g. glucose oxidase, lipases, phospholipases and hexose oxidase, and proteases.

TECHNICAL EFFECT

The present invention is predicated upon the surprising finding that the lipid:carbohydrate acyltransferases according to the present invention can perform carbohydrate-esterification via alcoholosis, i.e. acyl transfer from a lipid, in a foodstuff with a significant water content. Prior art suggests that such enzymes if they would function at all in this manner would only function in a solvent environment (i.e. in environments with low or no water content).

The present invention may provide one or more of the following unexpected technical effects in dough and/or baked products: an improved specific volume of either the dough or the baked products (for example of bread and/or of cake); an improved dough stability; an improved crust score (for example a thinner and/or crispier bread crust), an improved crumb score (for example a more homogenous crumb distribution and/or a finer crumb structure and/or a softer crumb); an improved appearance (for example a smooth surface without blisters or holes or substantially without blisters or holes); a reduced staling; an enhanced softness; an improved odour; an improved taste.

Suitably, the present invention may provide one or more of the following unexpected technical effects in a foodstuff: an improved appearance, an improved mouthfeel, an improved stability, an improved taste, an improved softness, an improved resilience, an improved emulsification.

Suitably, the present invention may provide one or more of the following unexpected technical effects in dairy products, such as ice cream for example: an improved

mouthfeel (preferably a more creamy mouthfeel); an improved taste; an improved meltdown.

Suitably, the present invention may provide one or more of the following unexpected technical effects in egg or in egg products: improved stability of emulsion; thermal stability of emulsion; improved flavour; reduced mal-odour; improved thickening properties.

Suitably, the present invention may provide one or more of the following unexpected technical effects in cheese: a decrease in the oiling-off effect in cheese; an increase in cheese yield; an improvement in flavour; a reduced mal-odour; a reduced "soapy" taste.

The improvements observed with lipid:carbohydate acyltransferase according to the present invention are in comparison to when lipolytic enzymes without acyltransferase activity, such as triacylglycerol lipases and phospholipases, are used.

ADVANTAGES

The generation of two emulsifiers and/or a carbohydrate ester *in situ* from at least one constituent of the food material, means that the food material will contain at least one less additive material. This is advantageous because of the improvement in the ease of production. For example, no further processing or addition of ingredients or addition of emulsifiers may be required, as a foodstuff comprising the two emulsifiers and/or a carbohydrate ester is produced. Moreover, the foodstuff may contain less "additives". The reduction or elimination of "additives" is desirable to consumers and inclusion of additives often must be declared to the consumer in the ingredients listing on the foodstuff. Thus, the present invention is further advantageous.

Also advantageously the emulsification properties of the foodstuff are enhanced, leading to improved appearance and/or structure and/or handling properties without a negative impact on taste.

In addition, advantageously the effect of "overdosing" observed when using lipases *per se*, is effectively overcome by the addition of an enzyme in accordance with the present invention. This is due at least in part to the fact that free fatty acids are not produced or only produced to an insignificant degree when using the enzyme according to the present invention.

ISOLATED

In one aspect, preferably the polypeptide or protein for use in the present invention is in an isolated form. The term "isolated" means that the sequence is at least substantially free from at least one other component with which the sequence is naturally associated in nature and as found in nature.

PURIFIED

In one aspect, preferably the polypeptide or protein for use in the present invention is in a purified form. The term "purified" means that the sequence is in a relatively pure state – e.g. at least about 51% pure, or at least about 75%, or at least about 80%, or at least about 90% pure, or at least about 95% pure or at least about 98% pure.

CLONING A NUCLEOTIDE SEQUENCE ENCODING A POLYPEPTIDE ACCORDING TO THE PRESENT INVENTION

A nucleotide sequence encoding either a polypeptide which has the specific properties as defined herein or a polypeptide which is suitable for modification may be isolated from any cell or organism producing said polypeptide. Various methods are well known within the art for the isolation of nucleotide sequences.

For example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the polypeptide.

If the amino acid sequence of the polypeptide is known, labelled oligonucleotide probes may be synthesised and used to identify polypeptide-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known polypeptide gene could be used to identify polypeptide-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.

Alternatively, polypeptide-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing an enzyme inhibited by the polypeptide, thereby allowing clones expressing the polypeptide to be identified.

In a yet further alternative, the nucleotide sequence encoding the polypeptide may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beucage S.L. *et al* (1981) Tetrahedron Letters 22, p 1859-1869, or the method described by Matthes *et al* (1984) EMBO J. 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al* (Science (1988) 239, pp 487-491).

NUCLEOTIDE SEQUENCES

The present invention also encompasses nucleotide sequences encoding polypeptides having the specific properties as defined herein. The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or antisense strand.

The term "nucleotide sequence" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence.

In a preferred embodiment, the nucleotide sequence *per se* encoding a polypeptide having the specific properties as defined herein does not cover the native nucleotide sequence in its natural environment when it is linked to its naturally associated sequence(s) that is/are also in its/their natural environment. For ease of reference, we shall call this preferred embodiment the "non-native nucleotide sequence". In this regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its native environment. Thus, the polypeptide of the present invention can be expressed by a nucleotide sequence in its native organism but wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

Preferably the polypeptide is not a native polypeptide. In this regard, the term "native polypeptide" means an entire polypeptide that is in its native environment and when it has been expressed by its native nucleotide sequence.

Typically, the nucleotide sequence encoding polypeptides having the specific properties as defined herein is prepared using recombinant DNA techniques (i.e. recombinant DNA). However, in an alternative embodiment of the invention, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods

well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23 and Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

MOLECULAR EVOLUTION

Once an enzyme-encoding nucleotide sequence has been isolated, or a putative enzyme-encoding nucleotide sequence has been identified, it may be desirable to modify the selected nucleotide sequence, for example it may be desirable to mutate the sequence in order to prepare an enzyme in accordance with the present invention.

Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites.

A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151).

Instead of site directed mutagenesis, such as described above, one can introduce mutations randomly for instance using a commercial kit such as the GeneMorph PCR mutagenesis kit from Stratagene, or the Diversify PCR random mutagenesis kit from Clontech.

A third method to obtain novel sequences is to fragment non-identical nucleotide sequences, either by using any number of restriction enzymes or an enzyme such as Dnase I, and reassembling full nucleotide sequences coding for functional proteins. Alternatively one can use one or multiple non-identical nucleotide sequences and introduce mutations during the reassembly of the full nucleotide sequence.

Thus, it is possible to produce numerous site directed or random mutations into a nucleotide sequence, either *in vivo* or *in vitro*, and to subsequently screen for improved functionality of the encoded polypeptide by various means.

As a non-limiting example, In addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wildtype or other mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide.

The application of the above-mentioned and similar molecular evolution methods allows the identification and selection of variants of the enzymes of the present invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate

AMINO ACID SEQUENCES

The present invention also encompasses amino acid sequences of polypeptides having the specific properties as defined herein.

As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide".

The amino acid sequence may be prepared/isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques.

Suitably, the amino acid sequences may be obtained from the isolated polypeptides taught herein by standard techniques.

One suitable method for determining amino acid sequences from isolated polypeptides is as follows:

Purified polypeptide may be freeze-dried and 100 µg of the freeze-dried material may be dissolved in 50 µl of a mixture of 8 M urea and 0.4 M ammonium hydrogen carbonate, pH 8.4. The dissolved protein may be denatured and reduced for 15 minutes at 50°C following overlay with nitrogen and addition of 5 µl of 45 mM dithiothreitol. After cooling to room temperature, 5 µl of 100 mM iodoacetamide may be added for the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

135 µl of water and 5 µg of endoproteinase Lys-C in 5 µl of water may be added to the above reaction mixture and the digestion may be carried out at 37°C under nitrogen for 24 hours.

The resulting peptides may be separated by reverse phase HPLC on a VYDAC C18 column (0.46x15cm;10µm; The Separation Group, California, USA) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides may be re-chromatographed on a Develosil C18 column using the same solvent system, prior to N-terminal sequencing. Sequencing may be done using an Applied Biosystems 476A sequencer using pulsed liquid fast cycles according to the manufacturer's instructions (Applied Biosystems, California, USA).

SEQUENCE IDENTITY OR SEQUENCE HOMOLOGY

The present invention also encompasses the use of sequences having a degree of sequence identity or sequence homology with amino acid sequence(s) of a polypeptide having the specific properties defined herein or of any nucleotide sequence encoding such a polypeptide (hereinafter referred to as a "homologous sequence(s)"). Here, the term "homologue" means an entity having a certain homology with the subject amino

acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity".

The homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of the enzyme.

In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

In the present context, a homologous sequence is taken to include a nucleotide sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to a nucleotide sequence encoding a polypeptide of the present invention (the subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a

time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux *et al* 1984 Nuc. Acids Research 12 p387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al* 1999 Short Protocols in Molecular Biology, 4th Ed – Chapter 18), FASTA (Altschul *et al* 1990 J. Mol. Biol. 403-410) and the GENEWORKS suite of

comparison tools. Both BLAST and FASTA are available for offline and online searching (see Ausubel *et al* 1999, pages 7-58 to 7-60). However, for some applications, it is preferred to use the GCG Bestfit program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; FEMS Microbiol Lett 1999 177(1): 187-8 and tatiana@ncbi.nlm.nih.gov).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Alternatively, percentage homologies may be calculated using the multiple alignment feature in DNASIS™ (Hitachi Software), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), *Gene* 73(1), 237-244).

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and

glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z), diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine.

Replacements may also be made by unnatural amino acids.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

Nucleotide sequences for use in the present invention or encoding a polypeptide having the specific properties defined herein may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences.

The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences discussed herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular

homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction polypeptide recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels,

or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

HYBRIDISATION

The present invention also encompasses sequences that are complementary to the sequences of the present invention or sequences that are capable of hybridising either to the sequences of the present invention or to sequences that are complementary thereto.

The term "hybridisation" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction (PCR) technologies.

The present invention also encompasses the use of nucleotide sequences that are capable of hybridising to the sequences that are complementary to the subject sequences discussed herein, or any derivative, fragment or derivative thereof.

The present invention also encompasses sequences that are complementary to sequences that are capable of hybridising to the nucleotide sequences discussed herein.

Hybridisation conditions are based on the melting temperature (Tm) of the nucleotide binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol. 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

Maximum stringency typically occurs at about Tm-5°C (5°C below the Tm of the probe); high stringency at about 5°C to 10°C below Tm; intermediate stringency at about 10°C to 20°C below Tm; and low stringency at about 20°C to 25°C below Tm. As will be understood by those of skill in the art, a maximum stringency hybridisation can be used to identify or detect identical nucleotide sequences while an intermediate (or low) stringency hybridisation can be used to identify or detect similar or related polynucleotide sequences.

Preferably, the present invention encompasses sequences that are complementary to sequences that are capable of hybridising under high stringency conditions or intermediate stringency conditions to nucleotide sequences encoding polypeptides having the specific properties as defined herein.

More preferably, the present invention encompasses sequences that are complementary to sequences that are capable of hybridising under high stringent conditions (e.g. 65°C

and 0.1xSSC {1xSSC = 0.15 M NaCl, 0.015 M Na-citrate pH 7.0}) to nucleotide sequences encoding polypeptides having the specific properties as defined herein.

The present invention also relates to nucleotide sequences that can hybridise to the nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

The present invention also relates to nucleotide sequences that are complementary to sequences that can hybridise to the nucleotide sequences discussed herein (including complementary sequences of those discussed herein).

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridising to the nucleotide sequences discussed herein under conditions of intermediate to maximal stringency.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under stringent conditions (e.g. 50°C and 0.2xSSC).

In a more preferred aspect, the present invention covers nucleotide sequences that can hybridise to the nucleotide sequences discussed herein, or the complement thereof, under high stringent conditions (e.g. 65°C and 0.1xSSC).

EXPRESSION OF POLYPEPTIDES

A nucleotide sequence for use in the present invention or for encoding a polypeptide having the specific properties as defined herein can be incorporated into a recombinant replicable vector. The vector may be used to replicate and express the nucleotide sequence, in polypeptide form, in and/or from a compatible host cell. Expression may be controlled using control sequences which include promoters/enhancers and other expression regulation signals. Prokaryotic promoters and promoters functional in eukaryotic cells may be used. Tissue specific or stimuli specific promoters may be

used. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

The polypeptide produced by a host recombinant cell by expression of the nucleotide sequence may be secreted or may be contained intracellularly depending on the sequence and/or the vector used. The coding sequences can be designed with signal sequences which direct secretion of the substance coding sequences through a particular prokaryotic or eukaryotic cell membrane.

EXPRESSION VECTOR

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

Preferably, the expression vector is incorporated in the genome of the organism. The term "incorporated" preferably covers stable incorporation into the genome.

The nucleotide sequence of the present invention or coding for a polypeptide having the specific properties as defined herein may be present in a vector, in which the nucleotide sequence is operably linked to regulatory sequences such that the regulatory sequences are capable of providing the expression of the nucleotide sequence by a suitable host organism, i.e. the vector is an expression vector.

The vectors of the present invention may be transformed into a suitable host cell as described below to provide for expression of a polypeptide having the specific properties as defined herein.

The choice of vector, e.g. plasmid, cosmid, virus or phage vector, will often depend on the host cell into which it is to be introduced.

The vectors may contain one or more selectable marker genes – such as a gene which confers antibiotic resistance e.g. ampicillin, kanamycin, chloramphenicol or tetracyclin

resistance. Alternatively, the selection may be accomplished by co-transformation (as described in WO91/17243).

Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

Thus, in a further embodiment, the invention provides a method of making nucleotide sequences of the present invention or nucleotide sequences encoding polypeptides having the specific properties as defined herein by introducing a nucleotide sequence into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector.

The vector may further comprise a nucleotide sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

REGULATORY SEQUENCES

In some applications, a nucleotide sequence for use in the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined herein may be operably linked to a regulatory sequence which is capable of providing for the expression of the nucleotide sequence, such as by the chosen host cell. By way of example, the present invention covers a vector comprising the nucleotide sequence of the present invention operably linked to such a regulatory sequence, i.e. the vector is an expression vector.

The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

Enhanced expression of the nucleotide sequence encoding the enzyme having the specific properties as defined herein may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions.

Preferably, the nucleotide sequence of the present invention may be operably linked to at least a promoter.

Examples of suitable promoters for directing the transcription of the nucleotide sequence in a bacterial, fungal or yeast host are well known in the art.

CONSTRUCTS

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes a nucleotide sequence encoding a polypeptide having the specific properties as defined herein for use according to the present invention directly or indirectly attached to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the Sh1-intron or the ADH intron, intermediate the promoter and the nucleotide sequence of the present invention. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In some cases, the terms do not cover the natural combination of the nucleotide sequence coding for the protein ordinarily associated with the wild type gene promoter and when they are both in their natural environment.

The construct may even contain or express a marker which allows for the selection of the genetic construct.

For some applications, preferably the construct comprises at least a nucleotide sequence of the present invention or a nucleotide sequence encoding a polypeptide having the specific properties as defined herein operably linked to a promoter.

HOST CELLS

The term "host cell" - in relation to the present invention includes any cell that comprises either a nucleotide sequence encoding a polypeptide having the specific properties as defined herein or an expression vector as described above and which is used in the recombinant production of a polypeptide having the specific properties as defined herein.

Thus, a further embodiment of the present invention provides host cells transformed or transfected with a nucleotide sequence of the present invention or a nucleotide sequence that expresses a polypeptide having the specific properties as defined herein. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells. Preferably, the host cells are not human cells.

Examples of suitable bacterial host organisms are gram negative bacterium or gram positive bacteria.

Depending on the nature of the nucleotide sequence encoding a polypeptide having the specific properties as defined herein, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or other fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a different fungal host organism should be selected.

The use of suitable host cells, such as yeast, fungal and plant host cells – may provide for post-translational modifications (e.g. myristoylation, glycosylation, truncation, lapidation and tyrosine, serine or threonine phosphorylation) as may be needed to confer optimal biological activity on recombinant expression products of the present invention.

The host cell may be a protease deficient or protease minus strain.

ORGANISM

The term "organism" in relation to the present invention includes any organism that could comprise a nucleotide sequence according to the present invention or a nucleotide sequence encoding for a polypeptide having the specific properties as defined herein and/or products obtained therefrom.

Suitable organisms may include a prokaryote, fungus, yeast or a plant.

The term "transgenic organism" in relation to the present invention includes any organism that comprises a nucleotide sequence coding for a polypeptide having the specific properties as defined herein and/or the products obtained therefrom, and/or wherein a promoter can allow expression of the nucleotide sequence coding for a polypeptide having the specific properties as defined herein within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, a nucleotide sequence coding for a polypeptide having the specific properties as defined herein, constructs as defined herein, vectors as defined herein, plasmids as defined herein, cells as defined herein, or

the products thereof. For example the transgenic organism can also comprise a nucleotide sequence coding for a polypeptide having the specific properties as defined herein under the control of a heterologous promoter.

TRANSFORMATION OF HOST CELLS/ORGANISM

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*.

Teachings on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook *et al* (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press). If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

In another embodiment the transgenic organism can be a yeast.

Filamentous fungi cells may be transformed using various methods known in the art – such as a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known. The use of *Aspergillus* as a host microorganism is described in EP 0 238 023.

Another host organism can be a plant. A review of the general techniques used for transforming plants may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). Further teachings on plant transformation may be found in EP-A-0449375.

General teachings on the transformation of fungi, yeasts and plants are presented in following sections.

TRANSFORMED FUNGUS

A host organism may be a fungus - such as a filamentous fungus. Examples of suitable such hosts include any member belonging to the genera Thermomyces, Acremonium, Aspergillus, Penicillium, Mucor, Neurospora, Trichoderma and the like.

Teachings on transforming filamentous fungi are reviewed in US-A-5741665 which states that standard techniques for transformation of filamentous fungi and culturing the fungi are well known in the art. An extensive review of techniques as applied to *N. crassa* is found, for example in Davis and de Serres, *Methods Enzymol* (1971) 17A: 79-143.

Further teachings on transforming filamentous fungi are reviewed in US-A-5674707.

In one aspect, the host organism can be of the genus *Aspergillus*, such as *Aspergillus niger*.

A transgenic *Aspergillus* according to the present invention can also be prepared by following, for example, the teachings of Turner G. 1994 (Vectors for genetic manipulation. In: Martinelli S.D., Kinghorn J.R.(Editors) *Aspergillus*: 50 years on. Progress in industrial microbiology vol 29. Elsevier Amsterdam 1994. pp. 641-666).

Gene expression in filamentous fungi has been reviewed in Punt *et al.* (2002) Trends Biotechnol 2002 May;20(5):200-6, Archer & Peberdy Crit Rev Biotechnol (1997) 17(4):273-306.

TRANSFORMED YEAST

In another embodiment, the transgenic organism can be a yeast.

A review of the principles of heterologous gene expression in yeast are provided in, for example, *Methods Mol Biol* (1995), 49:341-54, and *Curr Opin Biotechnol* (1997) Oct;8(5):554-60

In this regard, yeast – such as the species *Saccharomyces cerevisi or Pichia pastoris* (see FEMS Microbiol Rev (2000 24(1):45-66), may be used as a vehicle for heterologous gene expression.

A review of the principles of heterologous gene expression in *Saccharomyces cerevisiae* and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a vehicle for the expression of heterologous genes", *Yeasts*, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

For the transformation of yeast, several transformation protocols have been developed. For example, a transgenic Saccharomyces according to the present invention can be prepared by following the teachings of Hinnen *et al.*, (1978, *Proceedings of the National Academy of Sciences of the USA* 75, 1929); Beggs, J D (1978, *Nature*, London, 275, 104); and Ito, H *et al* (1983, J Bacteriology 153, 163-168).

The transformed yeast cells may be selected using various selective markers – such as auxotrophic markers dominant antibiotic resistance markers.

TRANSFORMED PLANTS/PLANT CELLS

A host organism suitable for the present invention may be a plant. A review of the general techniques may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

SECRETION

Often, it is desirable for the polypeptide to be secreted from the expression host into the culture medium from where the enzyme may be more easily recovered. According to the present invention, the secretion leader sequence may be selected on the basis of the desired expression host. Hybrid signal sequences may also be used with the context of the present invention.

Typical examples of heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*glaA* - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the a-factor gene (yeasts e.g. *Saccharomyces, Kluyveromyces* and *Hansenula*) or the α-amylase gene (*Bacillus*).

DETECTION

A variety of protocols for detecting and measuring the expression of the amino acid sequence are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS).

A wide variety of labels and conjugation techniques are known by those skilled in the art and can be used in various nucleic and amino acid assays.

A number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for these procedures.

Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles and the like. Patents teaching the use of such labels include US-A-3,817,837; US-A-3,850,752; US-A-3,939,350; US-A-3,996,345; US-A-4,277,437; US-A-4,275,149 and US-A-4,366,241.

Also, recombinant immunoglobulins may be produced as shown in US-A-4,816,567.

FUSION PROTEINS

A polypeptide having the specific properties as defined herein may be produced as a fusion protein, for example to aid in extraction and purification thereof. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β-galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the protein sequence.

Gene fusion expression systems in *E. coli* have been reviewed in Curr. Opin. Biotechnol. (1995) 6(5):501-6.

In another embodiment of the invention, the amino acid sequence of a polypeptide having the specific properties as defined herein may be ligated to a heterologous sequence to encode a fusion protein. For example, for screening of peptide libraries for agents capable of affecting the substance activity, it may be useful to encode a chimeric substance expressing a heterologous epitope that is recognised by a commercially available antibody.

The invention will now be described, by way of example only, with reference to the following Figures and Examples.

Figure 1 shows a pfam00657.6 consensus sequence (SEQ ID No. 1);

Figure 2 shows an amino acid sequence (SEQ ID No. 2) obtained from the organism *Aeromonas hydrophila;*

Figure 3 shows an amino acid sequence (SEQ ID No. 3) obtained from the organism *Aeromonas salmonicida;*

Figure 4 shows an amino acid sequence (SEQ ID No. 4) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number NP_631558);

Figure 5 shows an amino acid sequence (SEQ ID No. 5) obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number: CAC42140);

Figure 6 shows an amino acid sequence (SEQ ID No. 6) obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number P41734);

Figure 7 shows an alignment of selected sequences to pfam00657.6 consensus sequence;

Figure 8 shows a pairwise alignment of SEQ ID No. 3 with SEQ ID No. 2 showing 93% amino acid sequence identity. The signal sequence is underlined. + denotes differences. The GDSX motif containing the active site serine 16, and the active sites aspartic acid 116 and histidine 291 are highlighted (see shaded regions). Numbers after the amino acid is minus the signal sequence;

Figure 9 shows a nucleotide sequence (SEQ ID No. 7) encoding a lipid:carbohydrate acyl transferase according to the present invention obtained from the organism *Aeromonas hydrophila;*

Figure 10 shows a nucleotide sequence (SEQ ID No. 8) encoding a lipid:carbohydrate acyl transferase according to the present invention obtained from the organism *Aeromonas salmonicida;*

Figure 11 shows a nucleotide sequence (SEQ ID No. 9) encoding a lipid:carbohydrate acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number NC_003888.1:8327480..8328367);

Figure 12 shows a nucleotide sequence (SEQ ID No. 10) encoding a lipid:carbohydrate acyl transferase according to the present invention obtained from the organism *Streptomyces coelicolor* A3(2) (Genbank accession number AL939131.1:265480..266367);

Figure 13 shows a nucleotide sequence (SEQ ID No. 11) encoding a lipid:carbohydrate acyl transferase according to the present invention obtained from the organism *Saccharomyces cerevisiae* (Genbank accession number Z75034);

Figure 14 shows an amino acid sequence (SEQ ID No. 12) obtained from the organism *Ralstonia* (Genbank accession number: AL646052);

Figure 15 shows a nucleotide sequence (SEQ ID No. 13) encoding a lipid:carbohydrate acyl transferase according to the present invention obtained from the organism *Ralstonia;* and

Figure 16 shows that homologues of the *Aeromonas* genes can be identified using the basic local alignment search tool service at the National Center for Biotechnology Information, NIH, MD, USA and the completed genome databases. The GDSX motif was used in the database search and a number of sequences/genes potentially encoding enzymes with lipolytic activity were identified. Genes were identified from the genus Streptomyces, Xanthomonas and Ralstonia. As an example below, the Ralstonia solanacearum was aligned to the Aeromonas salmonicida (satA) gene. Pairwise alignment showed 23% identity. The active site serine is present at the amino terminus and the catalytic residues histidine and aspartic acid can be identified.

EXAMPLES

Example 1 : Use of a lipid:carbohydrate acyltransferase during bread production.

One of the limitations of using lipases in bread making is that free fatty acid is formed during the lipase reaction. It is well known that formation of too much free fatty acid

will have a negative impact on the baking performance of flour, because the gluten gets too stiff and a bucky (i.e. a less elastic) dough is formed which can not expand during fermentation and baking.

Formation of free fatty acid should also be avoided from the point of oxidative stability, because free fatty acids are more prone to lipid oxidation than the corresponding triglyceride.

In the present invention the problems with free fatty acid formation when adding a lipolytic enzyme to a dough has been overcome by using a lipid:carbohydrate acyltransferase which, instead of producing free fatty acids, transfers one or more fatty acids from the lipid acyl donor to a sugar as acceptor molecule. The acceptor molecule in a dough may be one or more of glucose, sucrose or maltose and/or other carbohydrates normally available in a dough.

In the following experiments a lipid:carbohydrate acyltransferase is tested in mini scale baking experiments, and the lipid components from a fully proofed dough were extracted with water saturated butanol and analysed by HPLC and GLC analysis.

Materials and methods:

Enzymes:
Lipid:carbohydrate acyltransferase according to the present invention, 550 LATU/g
Grindamyl Exel 16, a commercial lipase from Danisco. 5000 LIPU/g
Flour: Sølvmel number 2001084

Mini baking test:
Flour, 50 gram, dry yeast 1.0 gram, glucose 0.8 gram, salt 0.8 gram, 50 ppm ascorbic acid and water 400 Brabender units is kneaded in a 50g Brabender mixing bowl for 5 minutes at 30°C. Resting time is 10 minutes at 34°C . After resting, the dough is scaled 15 gram per dough and then moulded on a special device where the dough is rolled between a wooden plate and a Plexiglas frame. The doughs are proofed in tins

for 45 minutes at 34°C, and baked in a Voss household oven for 8 minutes at 225°C. After baking the breads are cooled to ambient temperature and after 20 minutes the breads are scaled and the volume is determined by rape seed displacement method. The breads are also cut and the crumb and crust of the breads are evaluated.

Lipid extraction and fatty acid analyses:

10 g of fully proofed dough is immediately frozen and freeze-dried. The freeze-dried dough is milled in a coffee mill and passed through an 800 micron screen. 1.5 g of the freeze-dried dough is scaled in a 15ml centrifuge tube with screw lid. 7.5 ml of water saturated butanol (WSB) is added. The centrifuge tube is placed in a boiling water bath for 10 minutes. The tubes are placed in a Rotamix and turned 45 rpm for 20 minutes at ambient temperature and then placed in boiling water bath again for 10 minutes. The Rotamix is then turned on for 30 minutes at ambient temperature. The tubes were centrifuged at 3500 g for 5 minutes. 5ml of supernatant is transferred into a vial. WSB is evaporated to dryness under a steam of nitrogen, and analysed by HPLC and GLC. The free fatty acids in the extract are analysed as Cu-salts in isooctan measured at 715nm and quantified according to a calibration curve based on oleic acid. (Kwon, D.Y., and J.S., Rhee (1986) A Simple and Rapid Colourimetric Method for Determination of Free Fatty Acids for Lipase Assay, *JAOCS* 63:89).

HPLC analysis:

Column: LiChrospher 100 DIOL 5m (Merck art.16152) 250 x4.0 mm id with water jacket 50°C.

Mobil phase: (new line)

A:heptan/isopropanol/butanol/tetrahydrofuran/isooctan/H_2O*

64.5/17.5/7/5/5/1

B: isopropanol/butanol/tetrahydrofuran/isooctan/H_2O*

730/7/5/5/10

*1 mmol trifluoro acetic acid /l mobile phase

(pH=6.6 adjusted with NH_3)

Pump: Waters 510 + Gradient controller.

Gradient:	Flow: ml/min	Time: min	%A	%B
	1.0	0	100	0
	1.0	25	0	100
	1.0	30	0	100
	1.0	35	100	0
	1.0	40	100	0

Detector: CUNOW DDL21 (evaporative light-scattering)

Temp: 100 C. - volt:600 - air flow: 6.0 l/min

Injector: Hewlett Packard 1050. Injection volume: 50l

Sample preparation:

The wheat lipid is dissolved in 5ml $CHCl_3$ - CH_3OH (75-25) (sonicated for 10 min.) and filtered through 0.45m.

Calculation: Calibration curve for phosphatidylcholine (PC) (lecithin standard from the ILPS (International Lecithin and Phospholipid Society)) is used to calculate the amount of lipids and phospholipids.

Reference: Arnoldsson,K.C./ Kaufmann,P. Chromatographia Vol.38,5/6-1994, 317-324

Gas Chromatography:

Perkin Elmer 8420 Capillary Gas Chromatography equipped with WCOT fused silica column 12.5 m x 0,25 mm ID x 0,1µm 5%phenyl-methyl-silicone (CP Sil 8 CB from Crompack).

Carrier: Helium.

Injection: 1.5 µl with split.

Detector: FID. 385 °C.

Oven program:	1	2	3	4
Oven temperature, °C.	80	200	240	360
Isothermal, time, min	2	0	0	10
Temperature rate, °C. /min	20	10	12	

Sample preparation: 10 mg of wheat lipid is dissolved in 2ml heptane: pyridine 2:1 containing an internal standard heptadecane, 2 mg/ml. 500 µl of the sample is transferred to a crimp vial. 100 µl MSTFA(N-Methyl-N-trimethylsilyl-trifluoracetamid) is added and the reaction incubated for 15 minutes at 90°C.

Calculation: Response factors for mono-di-triglycerides, glucose ester and free fatty acid are determined from reference mixtures of these components. Based on these response factors the mono-di-triglycerides, glucose ester and free fatty acids in wheat lipids are calculated.

Lipase assay based on tributyrin as substrate (LIPU):

Lipase activity based on tributyrin may be measured according to Food Chemical Codex, Forth Edition, National Academy Press, 1996, p 803, with the modification that the sample is dissolved in deionized water instead of glycine buffer, and the pH stat set point is 5.5 instead of 7. 1 LIPU is defined as the quantity of enzyme which can liberate 1 µmol butyric acid per minute under assay conditions.

Lipid:carbohydrate acyltransferase assay for the determination of a lipid:carbohydrate acyltransferase unit, LATU(Gl):

Lipid acyl transferase activity is defined as the amount of µmol glucose ester formed per minute from lecithin as donor and glucose as acceptor molecule under assay conditions using the following procedure: Substrate :0,6% Avanti phospholipid(95% PC), 0.4 % glucose, 0.05M HEPES buffer pH 7 and 5 mM $CaCl_2$. Glucose is dissolved in buffer and Avanti phospholipid is dispersed by heating to 40°C and homogenized on a Turrax mixer for 10 seconds. 1 ml substrate is thermostated to 30 °C for 5 minutes. 100 µl of enzyme solution is added. After 10 minutes the reaction is stopped by adding 0.1 ml 1M HCl. The lipid components are extracted into an organic phase by adding 1 ml $CHCl_3$:CH_3OH 2:1. 500 µl of the organic phase is transferred to a 10 ml flask and evaporated at 60°C in a stream of nitrogen. The amount of glucose ester formed is determined by GLC analyses, using glucose monooleate (>95%) as standard.

Baking experiments:

Baking experiments are conduced according the mini baking test procedure with the addition of enzymes as shown in Table 1. The specific bread volume, crust and crumb are evaluated.

Table 1:

Experiment	Enzyme	Dosage per kg flour
1	Control (no enzyme)	
2	Lipid:carbohydrate acyltransferase	400 LATU(Gl)
3	Grindamyl Exel 16	500 LIPU

Fully proofed dough from this baking experiment are frozen and freeze-dried and the dough lipid extracted with water saturated butanol (WSB). The isolated dough lipids are analysed by GLC and HPLC.

Results and conclusion:

Preliminary results indicate that the lipid:carbohydrate acyltransferase clearly demonstrates a positive effect on both bread volume and bread appearance. In particular, preliminary results indicate that the use of the lipid:carbohydrate acyltransferase results in increased specific bread volume as compared with that obtained with the control (no enzyme) and that obtained with the use of a commercially available lipolytic enzyme, namely Grindamyl Exel 16.

In addition, preliminary results of the lipid analyses suggests that the lipid:carbohydrate acyltransferase transfers an acyl group from a lipid acyl donor to a carbohydrate acyl acceptor. In particular, the preliminary results indicate that the amount of free fatty acid produced following the use of the lipid:carbohydrate acyltransferase is only marginally increased compared with that of the control (no enzyme). In addition, the polar lipid PC is apparently converted to the corresponding monoester lysophosphatidylcholine (LPC). Preliminary results also indicated that glucose ester is formed in the dough containing the lipid:carbohydrate acyltransferase.

In contrast, no or an insignificant amount of glucose ester is formed when the lipolytic enzyme Grindamyl Exel 16 is used.

Example 2: Standard ice cream with dairy fat

Recipe:

	Negative control*	Positive control: with commercial emulsifier	With enzyme
Dairy cream, 38%	23.65	23.65	23.65
Skimmed milk	53.30	53.30	53.30
Skimmed milk powder	4.90	4.90	11.30
Sugar	12.00	12.00	12.00
Glucose syrup, DE 42, 75% TS	5.25	5.25	5.25
Stabiliser blend	0.2	0.2	0.2
Cremodan SE 30**		0.6	
Lipid:carbohydrate acyltransferase, 500 LATU(GI)/g			0.1
Grindsted Flavouring 2976***	0.1	0.1	0.1
Colour	+	+	+

* Negative control means no commercial emulsifier and no enzyme according to the present invention

**Cremodan SE 30 is a commercial mono-diglyceride E471 from Danisco A/S

***Grindsted Flavouring 2976 is a commercial flavour from Danisco A/S

Ice Cream production process:

1. Dairy cream and glucose syrup are heated to approx. 40°C The lipid:carbohydrate acyltransferase or the commercial emulsifier (if any) is added, and the mixture is reacted for 30 minutes. A sample is taken from each mixture for analysis.
2. The other liquid ingredients are heated to approx. 40°C.
3. The other dry ingredients are added. (stabiliser blend is mixed with sugar before addition).
4. When the dry ingredients are dissolved the dairy cream-glucose mixture is added.
5. The ice cream mix is pasteurised at 80-85°C/20-40 seconds.
6. And then homogenised at 80°C (190 bar).
7. The ice cream mix is cooled to ageing temperature, 4°C.
8. The ice cream is frozen in continuous freezer to 100% overrun.
9. The hardening process is done in a tunnel at -40°C.

Results:

Preliminary results indicated that both the ice cream produced with lipid:carbohydrate acyltransferase and the ice cream produced with emulsifier Cremodan SE 30 provided good taste and excellent creamy mouthfeel. Preliminary results indicate that the ice cream produced with the lipid:carbohydrate acyltransferase provided an improved or at least a comparable melt down compared with ice cream produced with the emulsifier Cremodan SE 30; both of which were improved as compared with the negative control.

The samples taken out during the ice cream production process (see step 1 thereof) are extracted with chloroform:methanol 2:1 and the lipid layer isolated and analysed by GLC and HPLC. Preliminary results indicate that the sample taken from the mixture comprising the lipid:carbohydrate acyltransferase contains, as well as one or more of the emulsifiers monoglyceride, glucose ester. In addition, preliminary results indicate that

the lipid:carbohydrate acyltransferase containing sample has one or more of the following as compared with the positive and negative controls: an increased amount of glucose ester, an increased amount of monoglyceride. In addition, preliminary results indicate that the lipid:carbohydrate acyltransferase containing sample has less triacylglyceride that samples taken from the negative and positive controls.

Example 3: Standard Ice Cream with vegetable fat

Recipe:

	Negative control*	Positive control: with emulsifier	With enzyme
Skimmed milk powder	11.30	11.30	11.30
Vegetable fat (HCO)	8.00	8.00	8.00
Sugar	12.00	12.00	12.00
Glucose syrup, DE 42, 75% TS	5.25	5.25	5.25
Stabiliser blend	0.2	0.2	0.2
Cremodan SE 30**		0.6	
Lipid acyl transferase, 500 LATU(GL)/g			0.1
Grindsted Flavouring 2976***	0.1	0.1	0.1
Colour	+	+	+
Water	62.55	62.55	62.55

* Negative control means no commercial emulsifier and no enzyme according to the present invention

**Cremodan SE 30 is a commercial mono-diglyceride E471 from Danisco A/S

***Grindsted Flavouring 2976 is a commercial flavour from Danisco A/S

Ice cream production process:

1. The skim milk powder, vegetable fat, glucose syrup and the water are heated to 40°C. The lipid:carbohydrate acyltransferase or commercial emulsifier (if any) is added and the mixture is kept at 40°C for 30 minutes.
2. The other dry ingredients are added and dissolved. (The stabiliser blend is mixed with sugar before addition).
3. The ice cream mix is pasteurised at 80-85°C/20-40 seconds.
4. And then homogenised at 80°C (190 bar).
5. The ice cream mix is cooled to ageing temperature, 4°C.
6. The ice cream is frozen in continuous freezer to 100% overrun.
7. The hardening process is done in a tunnel at -40°C.

Results:

Both tests with Cremodan SE 30 (the commercial emulsifier) and with lipid:carbohydrate acyltransferase produced ice cream with good taste and excellent creamy mouthfeel. Preliminary results indicate that the ice cream produced with the lipid:carbohydrate acyltransferase provided an improved or at least a comparable melt down compared with ice cream produced with the emulsifier Cremodan SE 30; both of which were improved as compared with the negative control.

Example 4: Lipid:carbohydrate acyltransferase for production of Pound cake

Pound cake recipe:

	1	2	3
Ingredients	Negative Control*	Positive Control* with commercial	Enzyme

		Emulsifier	
Sugar/Dextrose 5/1	250	250	250
Margarine	200	200	200
Soya oil	20	20	20
Egg	250	250	250
Wheat starch	110	110	110
Flour	120	120	120
Gatodan 504 cake gel**		15	
Lipid:carbohydrate acyltransferase, 500 ATU(Gl)/g			1
Baking powder	5	5	5
Flavouring	3	3	3

* Negative control means that it contains neither commercial emulsifier nor the enzyme according to the present invention.

**Gatodan 504 cake gel is a cake improver containing monoglyceride and polyglycerolester

Procedure:

Mixing:

Egg, oil and dextrose are transferred to a mixing bowl. Lipid:carbohydrate acyltransferase or emulsifier (if any) is added and agitated for 10 minutes.

The other ingredients are then added and the cake batter is mixed on a Hobart Mixer for 2 minutes in 1st gear and for 3 minutes in 3rd gear.

Baking:

350g cake batter is scaled in a cake tin and baked at 180°C for approx. 45 min. After baking the cakes are cooled to ambient and specific cake volume (ml/g) is measured. Cake appearance and crumb structure is evaluated subjectively.

After baking the cake crumb is frozen and freeze-dried. The freeze-dried cake crumb is grounded in a mill, sieved and extracted with $CHCl_3$:CH_3OH 2:1. Emulsifiers in the lipid phase are analysed by HPLC and GLC.

Results and conclusion:

Preliminary results with lipid:carbohydrate acyltransferase in pound cake show that the cakes produced with the enzyme have better specific cake volume, a better appearance (i.e. a smoother surface with less blisters and/or holes and a taller cake), and a better crumb structure (i.e. more homogenous and/or a more tender crumb) as compared with the negative control. The results achieved were almost comparable with those obtained using the conventional cake improver (Gatodan 504).

Preliminary results further indicate that the cake produced using the lipid:carbohydrate acyltransferase had an increased amount of monoglyceride and lysophosphatidylcholine compared with either the positive or negative control, indicating that the enzyme catalyses the formation of lysolecithin and monoglyceride which in a cake batter improves the aeration of the cake batter and stabilises the air cells. The preliminary results also indicate that the amount of sugar ester is increased in the cake produced using the lipid:carbohydrate acyltransferase as compared with either the positive or negative control.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A method for the *in situ* production of two emulsifiers in a foodstuff, wherein a lipid:carbohydrate acyltransferase is added to the foodstuff.

2. A method according to claim 1 wherein one of the emulsifiers is a carbohydrate ester.

3. A method for the *in situ* production of a carbohydrate ester in a foodstuff, wherein a lipid:carbohydrate acyltransferase is added to the foodstuff.

4. A method according to any one of claims 1-3 wherein the lipid:carbohydrate acyltransferase is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

5. A method according to claim 4 wherein the lipid:carbohydrate acyltransferase enzyme comprises His-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2.

6. A method according to any one of the preceding claims wherein the lipid:carbohydrate acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas*, *Streptomyces*, *Saccharomyces*, *Lactococcus*, *Mycobacterium*, *Streptococcus*, *Lactobacillus*, *Desulfitobacterium*, *Bacillus*, *Campylobacter*, *Vibrionaceae*, *Xylella*, *Sulfolobus*, *Aspergillus*, *Schizosaccharomyces*, *Listeria*, *Neisseria*, *Mesorhizobium* and *Candida*.

7. A method according to any one of the preceding claims wherein the lipid:carbohydrate acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid sequence shown as SEQ ID No. 12; (vii) an amino acid sequence which has

75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6 or SEQ ID No. 12.

8. A method according to claim 1 or claim 2 or any claim dependent upon claim 1 or claim 2, wherein the second emulsifier is one or more of the following: a monoglyceride or a lysophosphatidylcholine.

9. Use of a lipid:carbohydrate acyltransferase to prepare from a food material a foodstuff comprising at least two emulsifiers, wherein the two emulsifiers are generated from constituents of the food material by the lipid:carbohydrate acyltransferase.

10. Use according to claim 9 wherein one of the emulsifiers is a carbohydrate ester.

11. Use according to claim 9 or claim 10 wherein the lipid:carbohydrate acyltransferase is characterised as an enzyme which possesses acyl transferase activity and which comprises the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues L, A, V, I, F, Y, H, Q, T, N, M or S.

12. Use according to any one of claim 9-11 wherein the lipid:carbohydrate acyltransferase enzyme comprises His-309 or comprises a histidine residue at a position corresponding to His-309 in the amino acid sequence of the *Aeromonas hydrophila* lipolytic enzyme shown as SEQ ID No. 2.

13. Use according to any one of the preceding claims wherein the lipid:carbohydrate acyltransferase is obtainable from an organism from one or more of the following genera: *Aeromonas, Streptomyces, Saccharomyces, Lactococcus, Mycobacterium, Streptococcus, Lactobacillus, Desulfitobacterium, Bacillus, Campylobacter, Vibrionaceae, Xylella, Sulfolobus, Aspergillus, Schizosaccharomyces, Listeria, Neisseria, Mesorhizobium* and *Candida*.

14. Use according to any one of the preceding claims wherein the lipid:carbohydrate acyltransferase comprises one or more of the following amino acid sequences: (i) the amino acid sequence shown as SEQ ID No. 2; (ii) the amino acid sequence shown as SEQ ID No. 3; (iii) the amino acid sequence shown as SEQ ID No. 4; (iv) the amino acid sequence shown as SED ID No. 5; (v) the amino acid sequence shown as SEQ ID No. 6; (vi) the amino acid

sequence shown as SEQ ID No. 12, (vii) an amino acid sequence which has 75% or more identity with any one of the sequences shown as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6 or SEQ ID No. 12.

15. Use according to any one of the preceding claims, wherein the second emulsifier is one or more of the following: a monoglyceride or a lysophosphatidylcholine.

16. A food stuff obtainable by the method according to any one of claims 1-8.

ABSTRACT

METHOD

A method for the *in situ* production of two emulsifiers in a foodstuff, wherein a lipid:carbohydrate acyltransferase is added to the foodstuff. One of the emulsifiers is suitably a carbohydrate ester.

Figure 1

SEQ ID No. 1

```
  1 ivafGDSlTd geayygdsdg ggwgagladr Ltallrlrar prgvdvfnrg isGrtsdGrl
 61 ivDalvallF laqslglpoL pPYLsgdflr GANFAsagAt Ilptsgpfli QvqFkdfksq
121 vlelrqalgl lqellrllpv ldakspdlvt imiGtNilit saffgpkste sdrmvsvpef
181 kdnlrqlikr Lrsnngarii vlitlvilnl gplGClPlkl alalassknv dasgclerln
241 eavadfneal relaiskled qlrkdglpdv kgadvpyvDl ysifqdldgi qnpsayvyGP
301 ettkaCCGyG gryNynrvCG naglcnvtak aCnpssylls flfwDgfEps ekGykavAea
361 l
```

Figure 2

SEQ ID No. 2

```
  1 mkkwfvcllg lvaltvqaad srpafsrivm fgdslsdtgk myskmrgylp ssppyyegrf
 61 sngpvwleql tnefpgltia neaeggptav aynkiswnpk yqvinnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakeill fnlpdlgqnp
181 sarsqkvvea ashvsayhnq lllnlarqla ptgmvklfei dkqfaemlrd pqnfglsdqr
241 nacyggsyvw kpfasrsast dsqlsafnpq erlaiagnpl laqavaspma srsastlnce
301 gkmfwdqvhp ttvvhaalse paatfiesqy eflah
```

Figure 3

SEQ ID No. 3

```
  1 mkkwfvcllg lialtvqaad trpafsrivm fgdslsdtgk myskmrgylp ssppyyegrf
 61 sngpvwleql tkqfpgltia neaeggatav aynkiswnpk yqvynnldye vtqflqkdsf
121 kpddlvilwv gandylaygw nteqdakrvr daisdaanrm vlngakqill fnlpdlgqnp
181 sarsqkvvea vshvsayhnk lllnlarqla ptgmvklfei dkqfaemlrd pqnfglsdve
241 npcydggyvw kpfatrsvst drqlsafspq erlaiagnpl laqavaspma rrsasplnce
301 gkmfwdqvhp ttvvhaalse raatfietqy eflahg
```

Figure 4

SEQ ID No. 4

```
  1 mpkpalrrvm tatvaavgtl alglltdatah aapaqatptl dyvalgdsys agsgvlpvdp
 61 anllclrsta nyphviadtt garltdvtcg aaqtadftra qypgvapqld algtgtdlvt
121 ltiggndnst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgypwitpat adpscflklp laagdvpylr aiqahlndav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnalg errmaehtmd vlgld
```

Figure 5

SEQ ID No. 5

```
  1 mpkpalrrvm tatvaavgtl algltdatah aapaqatptl dyvalgdsys agsgvlpvdp
 61 anllclrsta nyphviadtt garltdvtcg aaqtadftra qypgvapqld algtgtdlvt
121 ltiggndnst finaitacgt agvlsggkgs pckdrhgtsf ddeieantyp alkeallgvr
181 arapharvaa lgypwitpat adpscflklp laagdvpylr aiqahlndav rraaeetgat
241 yvdfsgvsdg hdaceapgtr wiepllfghs lvpvhpnalg errmaehtmd vlgld
```

Figure 6

SEQ ID No. 6

```
  1 mdyekfllfg dsitefafnt rpiedgkdqy algaalvney trkmdilqrg fkgytsrwal
 61 kilpeilkhe snivmatifl gandacsagp qsvplpefid nirgmvslmk syhirpiiig
121 pglvdrekwe kekseeialg yfrtnenfai ysdalaklan eekvpfvaln kafqqeggda
181 wqqlltdglh fsgkgykifh dellkvietf ypqyhpkmmq yklkdwrdvl ddgsnims
```

Figure 7


Alignment of pfam00657.6 consensus sequence with P10480
P10480
Alignment of pfam00657.6 consensus sequence with AAG09804
AAG09804

Figure 7 cont'd

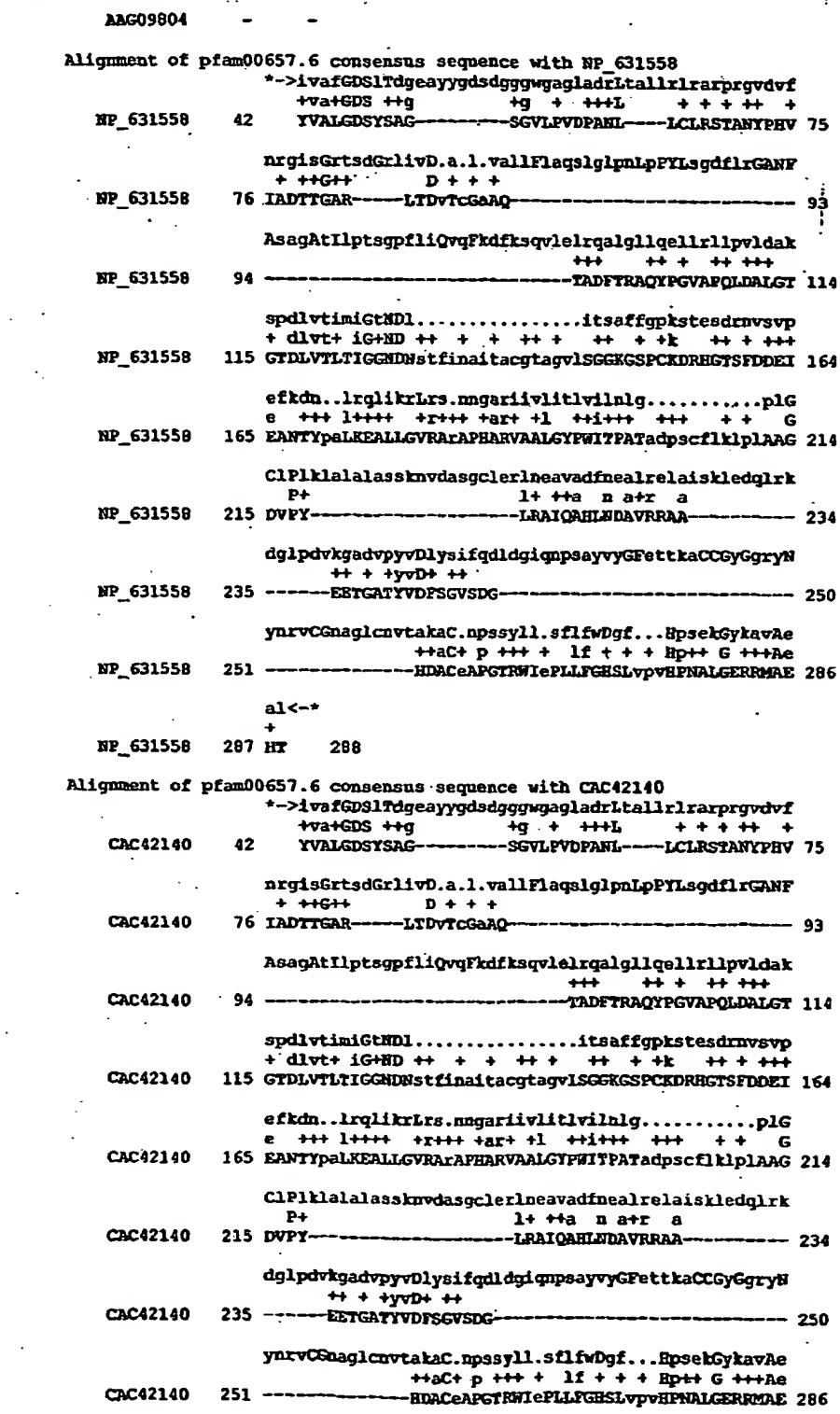


Figure 7 cont'd

```
                    al<-*
                    +
      CAC42140  287 HT    288

Alignment of pfam00657.6 consensus sequence with P41734
                   *->ivafGDSlTdg....eayygdsdgggwgagladrLtallrlrarprg
                        ++fGDS+T+   +++ + +  d+   ga+l + +        +r+
      P41734      6    FLLFGDSITEFafntRPIEDGKDQYALGAALVNEY---------TRK  43

                    vdvfnrgisGrtsdGrlivDalvallFlaqslglpnLpPYLsgdflrGAN
                    +d+  rg++G+t
      P41734     44 MDILQRGFKGYT-------------------------------------  55

                    FAsagAtIlptsgpfliQvqFkdfksqvlelrqalgllqellrllpvlda
                                                    +r+al++l+e+l+      +
      P41734     56 ------------------------------SRWALKILPEILKH-----E  70

                    kspdlvtimiGtNDlitsaffgpkstesdrnvsvpefkdnlrqlikrLrs
                     +  + ti++G+ND+          ++ +++ v++pef+dn+rq++++++s
      P41734     71 SNIVMATIFLGANDA---------CSAGPQSVPLPEFIDNIRQMVSLMKS 111

                    nngariivlitlvilnlgplGClPlklalalasslnvdasgclerlneav
                     ++++ii++++lv   ++              ++ k ++ +  + r+ne +
      P41734    112 YHIRPIIIGPGLVDREKN-------------EKEKSEKIALGYFRTNENF 148

                    adfnealrelaiskledqlrkdglpdvkgadvpyvDlysifqdldgiqnp
                    a +  al +la                 ++ +vp+v l+++fq+ +g++++
      P41734    149 AIYSDALAKLA----------------NEEKVPFVALNKAFQQDGGDAWQ 182

                    sayvyGFettkaCCGyGgryNynrvCGnaglcnvtakaCnpssyllslf1f
                    +                                                l+
      P41734    183 Q-----------------------------------------------LL 185

                    wDgfRpsekGykavAeal<-*
                     Dg+R+s kGyk+++++l
      P41734    186 TDGLRFSGKGYKIPHDEL    203
```

Figure 8


A.sal 1 MKKWFVCLLGLIALTVQAADTRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRF 60
A.hyd 1 MKKWFVCLLGLVALTVQAADSRPAFSRIVMFGDSLSDTGKMYSKMRGYLPSSPPYYEGRF 60
A. sal 61 SNGPVWLEQLTKQFPGLTIANEAEGGATAVAYNKISWNPKYQVINNLDYEVTQFLQKDSF 120
A. hyd 61 SNGPVWLEQLYNEFPGLTIANEAEGGPTAVAYNKISWNPKYQVINNLDYEVTQFLQKDSF 120
A. sal 121 KPDDLVILWVGANDYLAYGWNTEQDAKRVRDAISDAANRMVLNGAKQILLFNLPDLGQNP 180
A. hyd 121 KPDDLVILWVGANDYLAYGWNTEQDAKRVRDAISDAANRMVLNGAKEILLFNLPDLGQNP 180
A. sal 181 SARSQKVVEAVSHVSAYHNKLLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDVE 240
A.hyd 181 SARSQKVVEAASHVSAYHNQLLLNLARQLAPTGMVKLFEIDKQFAEMLRDPQNFGLSDQR 240
A. sal 241 NPCYDGGYVWKPFATRSVSTDRQLSAFSPQERLAIAGNPLLAQAVASPMARRSASPLNCE 300
A. hyd 241 NACYGGSYVWKPFASRSASTDSQLSAFNPQERLAIAGNPLLAQAVASPMAARSASTLNCE 300
A. sal 301 GKMFWDQVHPTTVVHAALSERAATFIETQYEFLAH 335
A. hyd 301 GKMFWDQVHPTTVVHAALSEPAATFIESQYEFLAH 335

Figure 9

```
   1  ATGAAAAAAT GGTTTGTGTG TTTATTGGGA TTGGTCGCGC TGACAGTTCA GGCAGCCGAC
  61  AGCCGTCCCG CCTTCTCCCG GATCGTGATG TTTGGCGACA GCCTCTCCGA TACCGGCAAG
 121  ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCCC CCTACTATGA GGGCCGCTTC
 181  TCCAACGGGC CCGTCTGGCT GGAGCAGCTG ACCAACGAGT TCCCGGGCCT GACCATAGCC
 241  AACGAGGCGG AAGGCGGACC GACCGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
 301  TATCAGGTCA TCAACAACCT GGACTACGAG GTCACCCAGT TCCTGCAAAA AGACAGCTTC
 361  AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGCGCCAACG ACTATCTGGC CTATGGCTGG
 421  AACACAGAGC AGGATGCCAA GCGGGTGCGC GACGCCATCA GCGATGCGGC CAACCGCATG
 481  GTGCTGAACG GCGCCAAGGA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCC
 541  TCGGCCCGCA GCCAGAAGGT GGTCGAGGCG GCCAGCCATG TCTCCGCCTA CCACAACCAG
 601  CTGCTGCTGA ACCTGGCACG CCAGCTGGCT CCCACCGGCA TGGTGAAGCT GTTCGAGATC
 661  GACAAGCAGT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACCAGAGG
 721  AACGCCTGCT ACGGTGGCAG CTATGTATGG AAGCCGTTTG CCTCCCGCAG CGCCAGCACC
 781  GACAGCCAGC TCTCCGCCTT CAACCCGCAG GAGCGCCTCG CCATCGCCGG CAACCCGCTG
 841  CTGGCCCAGG CCGTCGCCAG CCCCATGGCT GCCCGCAGCG CCAGCACCCT CAACTGTGAG
 901  GGCAAGATGT TCTGGGATCA GGTCCACCCC ACCACTGTCG TGCACGCCGC CCTGAGCGAG
 961  CCCGCCGCCA CCTTCATCGA GAGCCAGTAC GAGTTCCTCG CCCAC
```

Figure 10

```
   1  ATGAAAAAAT GGTTTGTTTG TTTATTGGGG TTGATCGCGC TGACAGTTCA GGCAGCCGAC
  61  ACTCGCCCCG CCTTCTCCCG GATCGTGATG TTCGGCGACA GCCTCTCCGA TACCGGCAAA
 121  ATGTACAGCA AGATGCGCGG TTACCTCCCC TCCAGCCCGC CCTACTATGA GGGCCGTTTC
 181  TCCAACGGAC CCGTCTGGCT GGAGCAGCTG ACCAAGCAGT TCCCGGGTCT GACCATCGCC
 241  AACGAAGCGG AAGGCGGTGC CACTGCCGTG GCTTACAACA AGATCTCCTG GAATCCCAAG
 301  TATCAGGTCT ACAACAACCT GGACTACGAG GTCACCCAGT TCTTGCAGAA AGACAGCTTC
 361  AAGCCGGACG ATCTGGTGAT CCTCTGGGTC GGTGCCAATG ACTATCTGGC ATATGGCTGG
 421  AATACGGAGC AGGATGCCAA GCGAGTTCGC GATGCCATCA GCGATGCGGC CAACCGCATG
 481  GTACTGAACG GTGCCAAGCA GATACTGCTG TTCAACCTGC CGGATCTGGG CCAGAACCCG
 541  TCAGCCCGCA GTCAGAAGGT GGTCGAGGCG GTCAGCCATG TCTCCGCCTA TCACAACAAG
 601  CTGCTGCTGA ACCTGGCACG CCAGCTGGCC CCCACCGGCA TGGTAAAGCT GTTCGAGATC
 661  GACAAGCAAT TTGCCGAGAT GCTGCGTGAT CCGCAGAACT TCGGCCTGAG CGACGTCGAG
 721  AACCCCTGCT ACGACGGCGG CTATGTGTGG AAGCCGTTTG CCACCCGCAG CGTCAGCACC
 781  GACCGCCAGC TCTCCGCCTT CAGTCCGCAG GAACGCCTCG CCATCGCCGG CAACCCGCTG
 841  CTGGCACAGG CCGTTGCCAG TCCTATGGCC CGCCGCAGCG CCAGCCCCCT CAACTGTGAG
 901  GGCAAGATGT TCTGGGATCA GGTACACCCG ACCACTGTCG TGCACGCAGC CCTGAGCGAG
 961  CGCGCCGCCA CCTTCATCGA GACCCAGTAC GAGTTCCTCG CCCACGGATG A
```

Figure 11

```
   1  ATGCCGAAGC CTGCCCTTCG CCGTGTCATG ACCGCGACAG TCGCCGCCGT CGGCACGCTC
  61  GCCCTCGGCC TCACCGACGC CACCGCCCAC GCCGCGCCCG CCCAGGCCAC TCCGACCCTG
 121  GACTACGTCG CCCTCGGCGA CAGCTACAGC GCCGGCTCCG GCGTCCTGCC CGTCGACCCC
 181  GCCAACCTGC TCTGTCTGCG CTCGACGGCC AACTACCCCC ACGTCATCGC GGACACGACG
 241  GGCGCCCGCC TCACGGACGT CACCTGCGGC GCCGCGCAGA CCGCCGACTT CACGCGGGCC
 301  CAGTACCCGG GCGTCGCACC CCAGTTGGAC GCGCTCGGCA CCGGCACGGA CCTGGTCACG
 361  CTCACCATCG GCGGCAACGA CAACAGCACC TTCATCAACG CCATCACGGC CTGCGGCACG
 421  GCGGGTGTCC TCAGCGGCGG CAAGGGCAGC CCCTGCAAGG ACAGGCACGG CACCTCCTTC
 481  GACGACGAGA TCGAGGCCAA CACGTACCCC GCGCTCAAGG AGGCGCTGCT CGGCGTCCGC
 541  GCCAGGGCTC CCCACGCCAG GGTGGCGGCT CTCGGCTACC CGTGGATCAC CCCGGCCACC
 601  GCCGACCCGT CCTGCTTCCT CAAGCTCCCC CTCGCCGCCG GTGACGTGCC CTACCTGCGG
 661  GCCATCCAGG CACACCTCAA CGACGCGGTC CGGCGGGCCG CCGAGGAGAC CGGAGCCACC
 721  TACGTGGACT TCTCCGGGGT GTCCGACGGC CACGACGCCT GCGAGGCCCC CGGCACCCGC
 781  TGGATCGAAC CGCTGCTCTT CGGGCACAGC CTCGTCCCG TCCACCCCAA CGCCCTGGGC
 841  GAGCGGCGCA TGGCCGAGCA CACGATGGAC GTCCTCGGCC TGGACTGA
```

Figure 12

```
   1  TCAGTCCAGG CCGAGGACGT CCATCGTGTG CTCGGCCATG CGCCGCTCGC CCAGGGCGTT
  61  GGGGTGGACG GGAACGAGGC TGTGCCCGAA GAGCAGCGGT TCGATCCAGC GGGTGCCGGG
 121  GGCCTCGCAG GCGTCGTGGC CGTCGGACAC CCCGGAGAAG TCCACGTAGG TGGCTCCGGT
 181  CTCCTCGGCG GCCCGCCGGA CCGCGTCGTT GAGGTGTGCC TGGATGGCCC GCAGGTAGGG
 241  CACGTCACCG GCGGCGAGGG GGAGCTTCAG GAAGCAGGAC GGGTCGGCGG TGGCCGGGGT
 301  GATCCACGGG TAGCCGAGAG CCGCCACCCT GGCGTGGGGA GCCCTGGCGC GGACGCCGAG
 361  CAGCGCCTCC TTGAGCGCGG GGTACGTGTT GGCCTCGATC TCGTCGTCGA AGGAGGTGCC
 421  GTGCCTGTCC TTGCAGGGGC TGCCCTTGCC GCCGCTGAGG ACACCCGCCG TGCCGCAGGC
 481  CGTGATGGCG TTGATGAAGG TGCTGTTGTC GTTGCCGCCG ATGGTGAGCG TGACCAGGTC
 541  CGTGCCGGTG CCGAGCGCGT CCAACTGGGG TGCGACGCCC GGGTACTGGG CCCGCGTGAA
 601  GTCGGCGGTC TGCGCGGCGC CGCAGGTGAC GTCCGTGAGG CGGGCGCCCG TCGTGTCCGC
 661  GATGACGTGG GGGTAGTTGG CCGTCGAGCG CAGACAGAGC AGGTTGGCGG GGTCGACGGG
 721  CAGGACGCCG GAGCCGGCGC TGTAGCTGTC GCCGAGGGCG ACGTAGTCCA GGGTCGGAGT
 781  GGCCTGGGCG GGCGCGGCGT GGGCGGTGGC GTCGGTGAGG CCGAGGGCGA GCGTGCCGAC
 841  GGCGGCGACT GTCGCGGTCA TGACACGGCG AAGGGCAGGC TTCGGCAT
```

Figure 13

```
   1  ATGGATTACG AGAAGTTTCT GTTATTTGGG GATTCCATTA CTGAATTTGC TTTTAATACT
  61  AGGCCCATTG AAGATGGCAA AGATCAGTAT GCTCTTGGAG CCGCATTAGT CAACGAATAT
 121  ACGAGAAAAA TGGATATTCT TCAAAGAGGG TTCAAAGGGT ACACTTCTAG ATGGGCGTTG
 181  AAAATACTTC CTGAGATTTT AAAGCATGAA TCCAATATTG TCATGGCCAC AATATTTTTG
 241  GGTGCCAACG ATGCATGCTC AGCAGGTCCC CAAAGTGTCC CCCTCCCCGA ATTTATCGAT
 301  AATATTCGTC AAATGGTATC TTTGATGAAG TCTTACCATA TCCGTCCTAT TATAATAGGA
 361  CCGGGGCTAG TAGATAGAGA GAAGTGGGAA AAAGAAAAAT CTGAAGAAAT AGCTCTCGGA
 421  TACTTCCGTA CCAACGAGAA CTTTGCCATT TATTCCGATG CCTTAGCAAA ACTAGCCAAT
 481  GAGGAAAAAG TTCCCTTCGT GGCTTTGAAT AAGGCGTTTC AACAGGAAGG TGGTGATGCT
 541  TGGCAACAAC TGCTAACAGA TGGACTGCAC TTTTCCGGAA AAGGGTACAA AATTTTTCAT
 601  GACGAATTAT TGAAGGTCAT TGAGACATTC TACCCCCAAT ATCATCCCAA AAACATGCAG
 661  TACAAACTGA AAGATTGGAG AGATGTGCTA GATGATGGAT CTAACATAAT GTCTTGA
```

Figure 14

(SEQ ID No. 12)

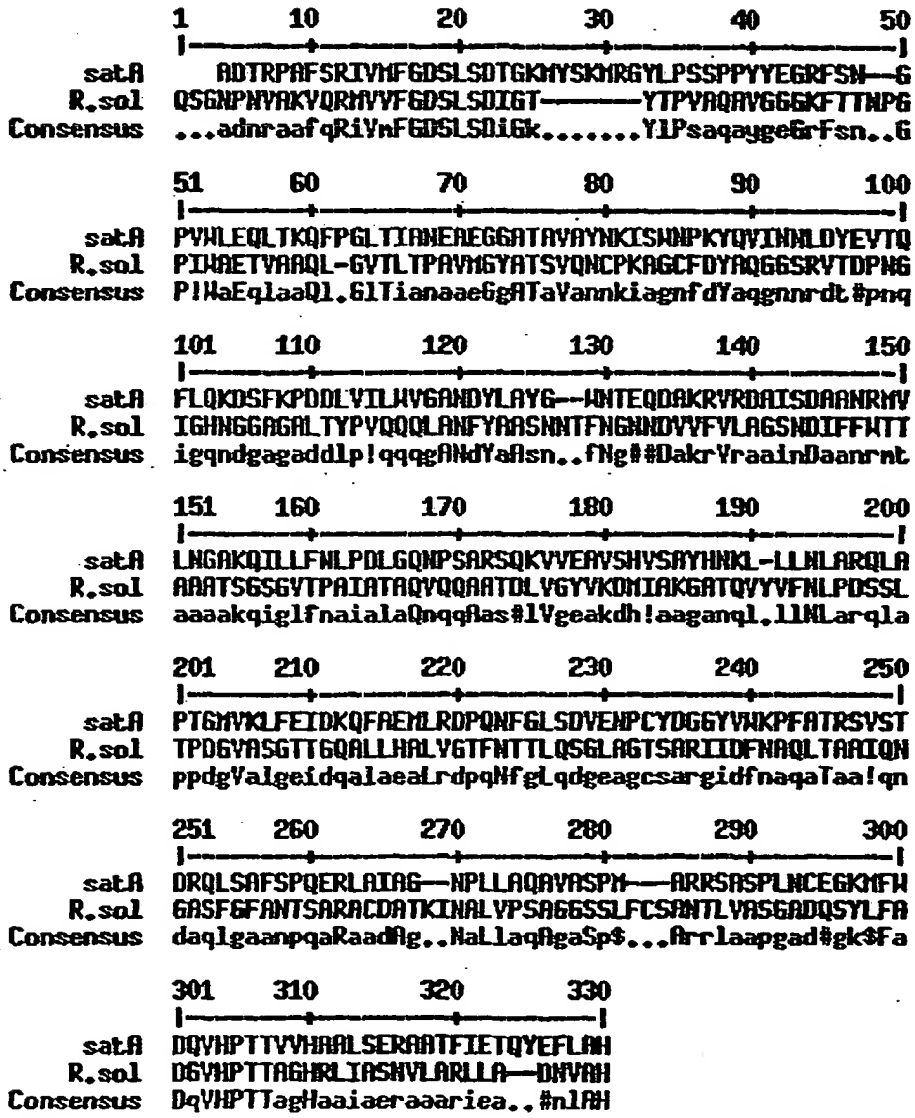
```
        10         20         30         40         50         60
         |          |          |          |          |          |
MNLRQWMGAA TAALALGLAA CGGGGTDQSG NPWVAKVQRW VVPGDSLSDI GTYTPVAQAV

        70         80         90        100        110        120
         |          |          |          |          |          |
GGGKFTTNPG PIWAETVAAQ LGVTLTPAVM GYATSVQNCP KAGCFDYAQG GSRVTDPNGI

       130        140        150        160        170        180
         |          |          |          |          |          |
GHNGGAGALT YPVQQQLANF YAASNNTFNG NNDVVFVLAG SNDIFFWTTA AATSGSGVTP

       190        200        210        220        230        240
         |          |          |          |          |          |
AIATAQVQQA ATDLVGYVKD MIAKGATQVY VFNLPDSSLT PDGVASGTTG QALLHALVGT

       250        260        270        280        290        300
         |          |          |          |          |          |
FNTTLQSGLA GTSARIIDFN AQLTAAIQNG ASFGFANTSA RACDATKINA LVPSAGGSSL

       310        320        330        340
         |          |          |          |
FCSANTLVAS GADQSYLFAD GVHPTTAGHR LIASNVLARL LADNVAH
```

Figure 15

(SEQ ID No. 13)

```
atgaacctgc gtcaatggat gggcgccgcc acggctgccc ttgccttggg cttggccgcg      60
tgcgggggcg gtgggaccga ccagagcggc aatcccaatg tcgccaaggt gcagcgcatg     120
gtggtgttcg gcgacagcct gagcgatatc ggcacctaca ccccgtcgc gcaggcggtg     180
ggcggcggca agttcaccac caacccgggc ccgatctggg ccgagaccgt ggccgcgcaa     240
ctgggcgtga cgctcacgcc ggcggtgatg ggctacgcca cctccgtgca gaattgcccc     300
aaggccggct gcttcgacta tgcgcagggc ggctcgcgcg tgaccgatcc gaacggcatc     360
ggccacaacg gcggcgcggg ggcgctgacc tacccggttc agcagcagct cgccaacttc     420
tacgcggcca gcaacaacac attcaacggc aataacgatg tcgtcttcgt gctggccggc     480
agcaacgaca ttttcttctg gaccactgcg gcggccacca gcggctccgg cgtgacgccc     540
gccattgcca cggcccaggt gcagcaggcc gcgacggacc tggtcggcta tgtcaaggac     600
atgatcgcca agggtgcgac gcaggtctac gtgttcaacc tgcccgacag cagcctgacg     660
ccggacggcg tggcaagcgg cacgaccggc caggcgctgc tgcacgcgct ggtgggcacg     720
ttcaacacga cgctgcaaag cgggctggcc ggcacctcgg cgcgcatcat cgacttcaac     780
gcacaactga ccgcggcgat ccagaatggc gcctcgttcg gcttcgccaa caccagcgcc     840
cgggcctgcg acgccaccaa gatcaatgcc ctggtgccga gcgccggcgg cagctcgctg     900
ttctgctcgg ccaacacgct ggtggcttcc ggtgcggacc agagctacct gttcgccgac     960
ggcgtgcacc cgaccacggc cggccatcgc ctgatcgcca gcaacgtgct ggcgcgcctg    1020
ctggcggata acgtcgcgca ctga                                           1044
```

Figure 16


1 10 20 30 40 50
satA ADTRPAFSRIVNFGDSLSDTGKMYSKMRGYLPSSPPYYEGRFSN--G
R.sol QSGNPNVNKVQRMVVFGDSLSDIGT-------YTPVNQRVGGGKFTTNPG
Consensus ...adnraafqRiVnFGDSLSDiGk.....YiPsaqaygeGrFsn..G
51 60 70 80 90 100
satA PVWLEQLTKQFPGLTIANEREGGATAVAYNKISWNPKYQVINNLDYEVTQ
R.sol PIWRETVRRQL-GVTLTPAVMGYATSVQNCPKAGCFDYAQGGSRVTDPNG
Consensus P!WaEqlaaQl.GlTianaaeGgATaVannkiagnfdYaqgnnrdt#pnq
101 110 120 130 140 150
satA FLQKDSFKPDDLVILWVGANDYLAYG--WNTEQDAKRVRDAISDAANRMV
R.sol IGHNGGAGALTYPVQQQLANFYAASNNTFNGWNDVVFVLAGSNDIFFWTT
Consensus igqndgagaddlp!qqqgANdYaAsn..fNg##DakrVraainDaanrnt
151 160 170 180 190 200
satA LNGAKQILLFNLPDLGQNPSARSQKVVEAVSHVSAYHNKL-LLNLARQLA
R.sol AAATSGSGVTPAIATAQVQQAATDLVGYVKDMIAKGATQVYVFNLPDSSL
Consensus aaaakqiglfnaialaQnqqAas#lVgeakdh!aaganql.llNLarqla
201 210 220 230 240 250
satA PTGMVKLFEIDKQFAEMLRDPQNFGLSDVENPCYDGGYVWKPFATRSVST
R.sol TPDGVASGTTGQALLHALVGTFNTTLQSGLAGTSARIIDFNAQLTAAIQN
Consensus ppdgVaIgeidqalaeaLrdpqNfgLqdgeagcsargidfnaqaTaa!qn
251 260 270 280 290 300
satA DRQLSAFSPQERLAIAG--NPLLAQAVASPN---ARRSASPLNCEGKMFW
R.sol GASFGFANTSARACDATKINALVPSAGGSSLFCSANTLVASGADQSYLFA
Consensus daqlgaanpqaRaadNg..NaLlaqNgaSp$...Arrlaapgad#gk$Fa
301 310 320 330
satA DQVHPTTVVHRALSERAATFIETQYEFLAH
R.sol DGVHPTTAGHRLIASHVLARLLA--DNVAH
Consensus DqVHPTTagHaaiaeraaariea..#nlAH